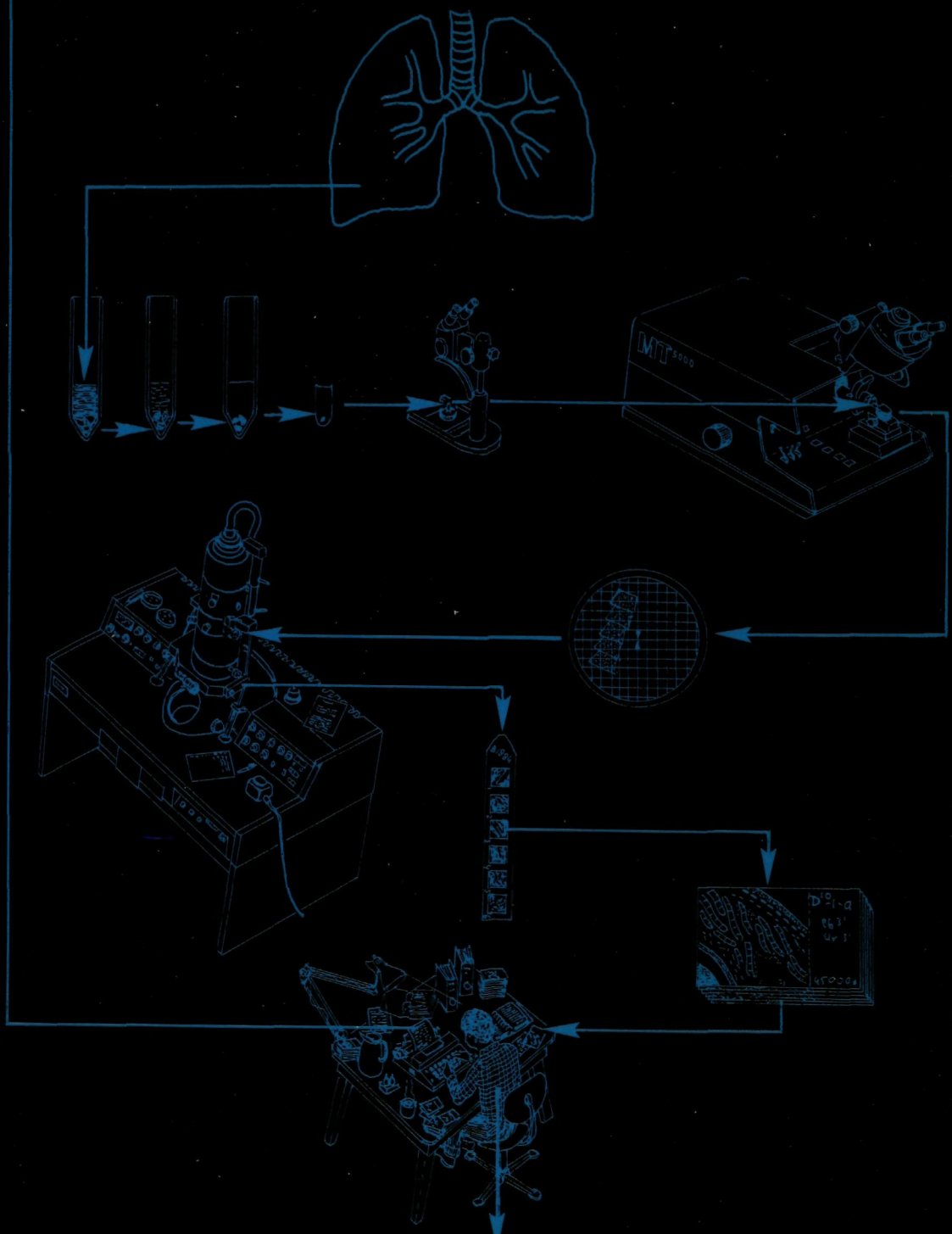
PROTEOGLYCANS OF LUNG PARENCHYMA

an electron microscopical and biochemical study



A. H. M. S. M. VAN KUPPEVELT

PROTEOGLYCANS OF LUNG PARENCHYMA

AN ELECTRON MICROSCOPICAL AND BIOCHEMICAL STUDY

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The investigations described in this thesis were carried out at the Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Nijmegen.

PROTEOGLYCANS OF LUNG PARENCHYMA
an electron microscopical and biochemical study

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B.M.F. VAN IERSEL VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 9 APRIL 1987 DES NAMIDDAGS TE 3.30 UUR.

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Antonius Henricus Minardus Severus Marie van Kuppevelt

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Prof. Dr. S.E. Wendelaar Bonga

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Aan Prof. Dr. Ch.M.A. Kuyper

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CHAPTER 1

General introduction.

Morphology

The primary function of the lung is to take care of a proper gas exchange, to supply the blood with O_2 and to remove CO_2. The lungs anatomy is constructed in such a way as to serve this function. Starting with the primary bronchi (originating from the trachea) there is a continuous branching of airways according to a pattern of irregular dichotomy, ultimately ending blindly. In total, an average of 23 generations of branching are present in a human lung (Weibel, 1980). The first 16 generations, ending with the terminal bronchioli, belong to the conducting zone; their function is to channal air into the terminal airway units. The terminal bronchioli split up into the respiratory brochioli, which already contain alveoli in their walls and they on their turn break up into the alveolar ducts from which the alveolar sacs and alveoli arise (Fig. 1). The 17th upto the 23rd generation comprise the transition and respiratory zone. All together these branches form the brochial tree. The pulmonary arteries are located adjacent to the air vessels and have a more or less similar branching pattern; pulmonary veins, however, follow a different pattern.

Lung parenchyma comprises, strictly speaking, only alveoli, alveolar sacs and alveolar ducts (Weibel, 1963). In general, however, with parenchyma is meant the tissue comprising the transition and the respiratory zone.

The actual gas exchange takes place in the alveoli (Fig. 2). On average there are 300 million alveoli in a human lung. Their form has been described as saucers, cups, brandy snifters and polyhedra. Literally alveolus is the Greek word for little cup. At high inflation levels their form can be compared with polydedra. The main part of an alveolus consists of the airspace, while the gas exchange process itself is located in the alveolar walls (alveolar septa). It should be noted that two alveoli have one septum in common.

Air transport in the lung is largely by flow; in the most peripheral airways, however, transport by diffusion becomes increasingly important, while the transport of alveolar air to the blood is basically a diffusional process. This implies that, in order to obtain an effective gas exchange, the barrier between the alveolar air and the blood must be extremely thin (Fig. 3). To facilitate gas exchange, alveolar cells are adapted. The most important cells of an alveolar septum are:

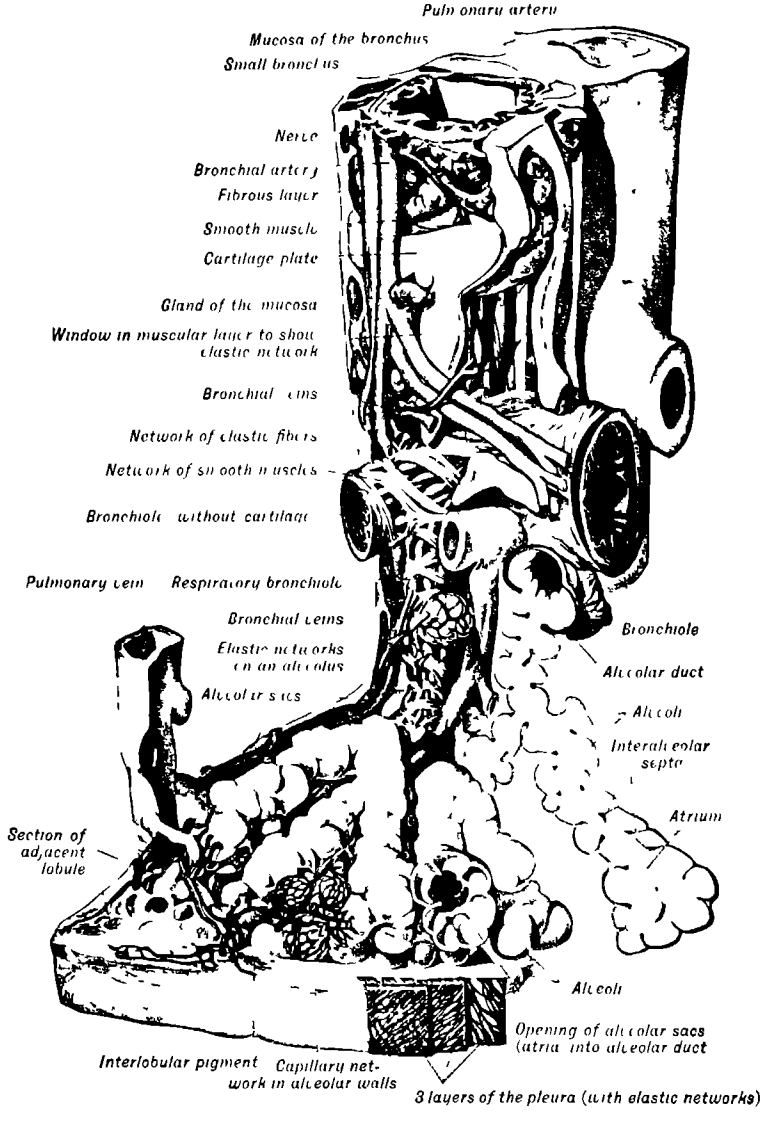

Fig. 1. Portion of the lung of a young man, showing branching from bronchus to alveoli. Bar: 0.3 mm. From Bloom, W. & Fawcett, D.W. 1975 in A Textbook of Histology, W.B. Saunders Co., Philidelphia-London-Toronto; with permission.

a) Type I epithelial cell (Fig. 3); this squamous alveolar cell contains a very thin layer of cytoplasm which can extend over a distance of about 50 µm from the nucleus. About 93% of the alveolar epithelial surface is formed by these cells (Crapo et al., 1982).

b) Capillary endothelial cells; these cells form the capillary surface and exist of a nucleus surrounded by a thin layer of cytoplasm which extends to thin sheets, similar to the type I epithelial cells. Numerous vesicles are frequently encountered in the endothelial cytoplasm taking care of transendothelial transport of macromolecules by the process of endo- and exocytosis.

The blood-air barrier is formed by the thin cytoplasmatic extensions of the epithelium and the endothelium and by a thin interstitium separating the two cell layers. In general this interstitium consists of the alveolar and the capillary basement membrane, which are in close apposition to each other. Basement membranes are continuous sheet-like structures found at the boundary between cells (endothelium, epithelium, muscle cells etc.) and connective tissue stroma. They form an extracellular scaffold and provide, amongst others, physical support.

c) Type II epithelial cell; this cuboidal cell has numerous short microvilli and is characterized by osmophilic, multilamellar bodies which contain phospholipids of which dipalmitoyl-phosphatidylcholine is a major component. The content of these bodies is secreted into the alveolar airspace, where it lines the tissue surface. This surfactant plays an important role in stabilizing the alveolus by reducing the surface tension, thereby preventing the alveolus from a collapse. Although the type II epithelial cells outnumber the type I epithelial cells, they constitute only about 7% of the epithelial surface (Crapo et al., 1982).

d) Fibroblast; this spindle-shaped cell is characteristic for the connective tissue. It contains large cytoplasmatic processes and synthesizes most components of the extracellular matrix (e.g. elastin, collagen, proteoglycans).

e) Alveolar macrophage (Fig. 4); this cell moves about on the alveolar surface. One of its functions is to phagocytose airborne particles (dust, bacteria) and to remove them from an alveolus. Alveolar macrophages are not permanently present.

There are other cell types less frequently encountered in an alveolar septum. Amongst them are the type III epithelial cell, of which the function is unknown (Dormans, 1985), the pericyte (a contractile cell, lining capillaries) and wandering blood cells (e.g. neutrophils). Cells making up lymphatic

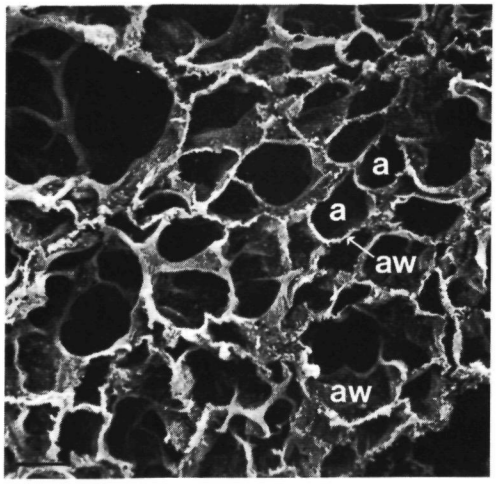

Fig. 2. Human lung alveoli. An alveolus consists of an alveolar airspace (a), limited by an alveolar wall (aw), in which the actual gas exchange takes place. Bar: 0.1 mm.

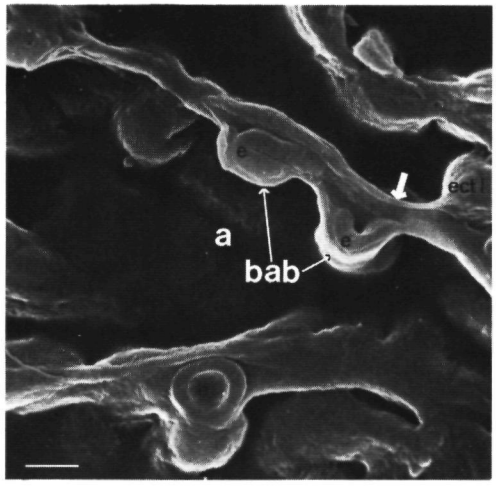

Fig. 3. Alveolar septa (mouse). The thin layer of tissue separating the blood from the alveolar airspace (a), is called the blood-air barrier (bab). It is formed by an epithelial cell layer a thin interstitium, and an endothelial cell layer. Furthermore, a type I epithelial cell (ect I) with a cytoplasmatic extension (arrow) can be seen. e: erythrocyte. Bar: 5 µm.

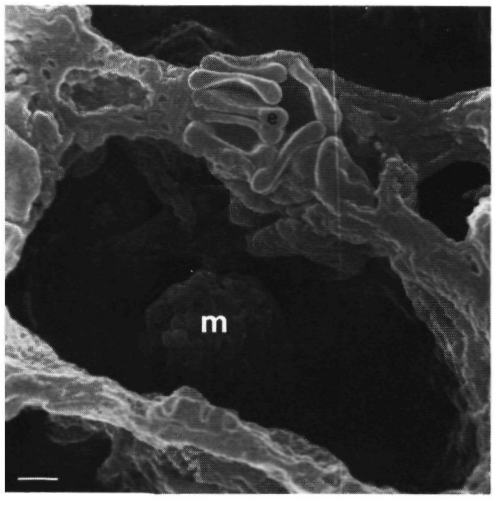

Fig. 4. Alveolus (mouse), containing an alveolar macrophage (m), attached to the alveolar surface. Capillaries form a major constituent of an alveolar wall. e: erythrocyte. Bar: 2.5 µm.

capillaries do not penetrate alveolar septa. Nerve fibres are small and scarce (Weibel, 1973).

Connective tissue

Since gas exchange is the basic function of the lung, there has to be a ventilatory system in order to supply fresh air and to remove "used" air. Upon inhalation the inspiratory muscles contract, the chest expands and the lungs are filled with air. In order to follow chest movements, the lung is attached to the chest wall by virtue of two sheets. One sheet, the visceral pleura, is fixed to the lung, while the other, the parietal pleura, is attached to the chest wall. Only a thin film of fluid is intercalated between the two pleuras and there exists a subatmospheric pressure between them. Upon expiration, when the inspiratory muscles relax, the lung retracts and air flows out of the lung. During quiet breathing this is a passive process and occurs as a result of the elastic recoil of the lung which is an intrinsic feature of the lung itself. The total surface available in a human lung for the actual gas exchange process is about 100-200 m^2. At the end of a deep breath only 20% of the total lung volume is tissue, including blood; 80% is air. Taking into account that the lung tissue has to follow volume changes during breathing, it is obvious that a flexible but stable framework should be on hand, especially to ensure the structural integrity of such delicate structures as alveoli.

The structural integrity of the lung depends on two major systems. One system is formed by the surfactant (see above) and will not be discussed further. The other is formed by the connective tissue framework, which main function is a supporting one. Connective tissue is characterized by its relatively limited number of cells (in lung the fibroblasts) and a large amount of extracellular matrix. Classically, the extracellular matrix is divided into two components: the fibrillar component (elastin fibers, collagen fibrils) and the ground substance, including proteoglycans. In lung, there exists a fibrous continuum of connective tissue elements extending from the airways all the way to the alveolar septa, as well as from the visceral pleura into the lung. The connective tissue in the alveolar walls is very delicate: capillaries constitute the largest part of an alveolar septum (Fig. 3 and 4). In the following, the three major components of the extracellular matrix, collagen, elastin and proteoglycans, will be discussed.

Collagen

In lung parenchyma about 20% of the dry weight is collagen (Clark et al., 1983). Collagens are glycoproteins composed of three α-chains, coiled into a triple helix conformation, making the molecule appear as a rod-like structure. Collagen has a typical amino acid composition: about 1/3 is glycine, about 10% is hydroxyproline, while also hydroxylysine is present. There are many types of collagen (types I-XI, Miller, 1985; Martin et al., 1985; Laurent, 1986) and in the light of recent reports (e.g. Yamauchi et al., 1986), other types are to be expected. The types I, II and III are the most abundant types; they are organized into fibrils which can be easily detected by electron microscopy. They show a characteristic banding pattern due to the distribution of collagen molecules in a fibril (quarter-staggered array). Of the other types of collagen, types IV and V are the best described. Type IV contains interruptions in the triple helix and seems to be restricted to basement membranes where it forms a fine network; type V collagen has a wider distribution. The other types of collagen are present in small quantities and most of them seem to have a restricted distribution (Mayne, 1984); they are at present poorly characterized.

Biochemical and/or immunocytochemical studies have shown that in lung the types I, II, III, IV, V and X are present (McLees et al., 1977; Madri & Furthmayr, 1979; Konomi et al., 1981; Sano et al., 1981; Dixit et al., 1981; Fujiwara & Nagai, 1981; Kelley, 1984; Raghu et al., 1985; Bearman et al., 1986). Collagen type I is the most abundant and constitutes about 65% of the total. Type II collagen is confined to the cartilagenous structures of the major airvessels; type X collagen has been demonstrated in smooth muscles lining alveolar ducts. The other types of collagen have been demonstrated in the parenchymal tissue, including alveoli. The major function of collagen in the lung is a supporting one. Collagen fibrils have a great tensile strength and may prevent the alveolus from overstretching. In general, however, the biological function of the different types of collagen is poorly understood.

Elastin

In lung parenchyma about 30% of the dry weight is elastin (Clark et al., 1983). Elastic fibers are composed of microfibrils and amorphous elastin. Microfibrils are believed to act as nucleation sites for elastin depostion. The elastin component is very hydrophobic in nature due to an abundance of nonpolar amino acids; it also contains unique amino acids such as desmosine and isodesmosine. In elastin the molecules are highly cross-linked. Elastin has the

remarkable property of being able to stretch to large extensions with little force and to return again to its original dimensions when the tension is released.

Proteoglycans and glycosaminoglycans

Structure

Proteoglycans are macromolecules which consist of a protein backbone to which glycosaminoglycan chains (s) and N-and/or O-linked oligosaccharides are covalently attached (Fig. 5). According to the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1986), they should be considered as a subclass of glycoproteins.

Glycosaminoglycans are linear polymers of repeating disaccharide units consisting of a hexosamine component and an uronic acid component; in keratan sulphate, the uronic acid is replaced by a galactose unit (Fig. 6). Due to variation in degree and place of sulphation, acetylation or sulphation of the amino group, type of glycosidic linkage, and geometry of the carboxylic and C-4 hydroxyl group, there exists an enormous potential of different glycosaminoglycans (and hence proteoglycans). Glycosaminoglycans can be grouped into 5 types: hyaluronic acid, chondroitin sulphate, dermatan sulphate, heparan sulphate/heparin and keratan sulphate (Fig. 6).

With the exception of hyaluronic acid, all these glycosaminoglycans are present in the tissue as proteoglycans. The attachment of glycosaminoglycans to the protein core involves a special linkage region generally consisting of serine-xylose-galactose-galactose-glucuronic acid; only keratan sulphate has an aberrant linkage region (Poole, 1986). There also exists a considerable variation in protein cores including such features as amino acid composition, molecular weight of the protein core and number of glycosaminoglycans attached (Hassell et al., 1986). On basis of their immunological properties and their sensitivity to trypsin protein cores can be grouped into different classes (Heingård et al., 1985). Proteoglycans and glycosaminoglycans are not rigid structures; NMR studies have shown that the protein core as well as the glycosaminoglycan side-chains are flexible structures (Brewer & Keiser, 1975; Torchia et al., 1977, 1981).

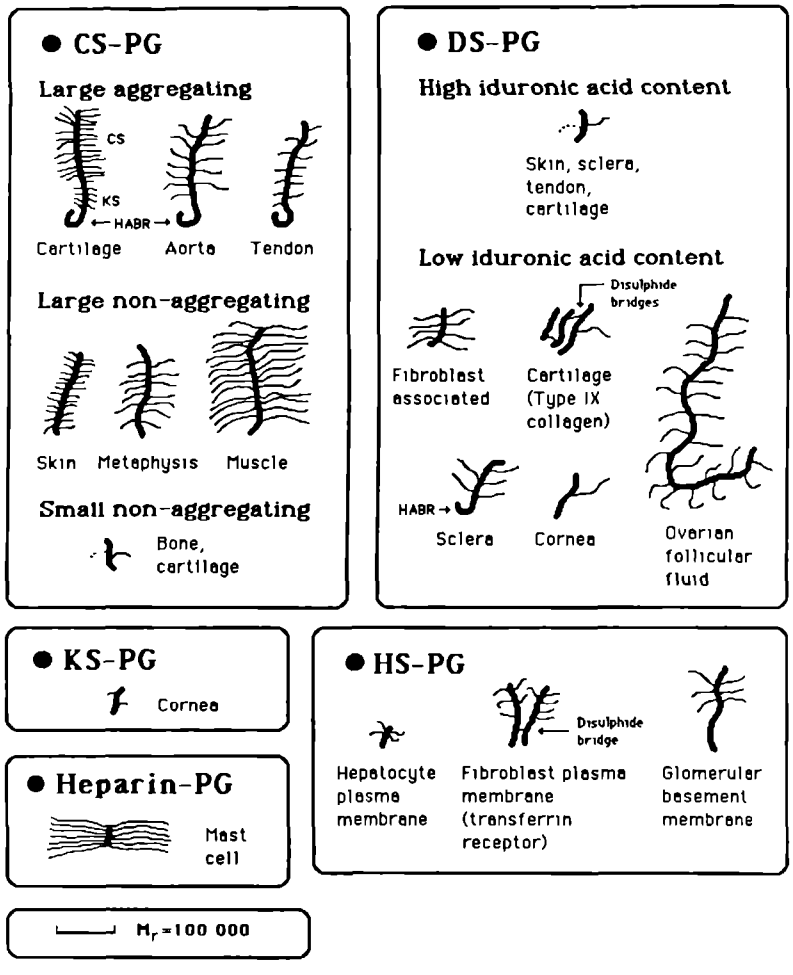

Fig. 5. Diagrammatic representation drawn to scale of some of the main proteoglycans (PGs), showing different types and diversity of structure. Oligosaccharides are not shown. The protein cores are drawn thicker than the attached glycosaminoglycans. CS: chondroitin sulphate; KS: keratan sulphate; DS: dermatan sulphate; HS: heparan sulphate; HABR: hyaluronic acid binding region. From Poole, A.R., 1986 in Biochemical Journal 236, p. 2, The Biochemical Society, London; with permission.

Location and function

Proteoglycans are found in nearly all mammalian tissues. Their main location is the extracellular matrix. Proteoglycans are not separated molecules. A number of interactions with other macromolecules such as collagen, fibronectin, laminin and elastin have been reported and are likely to be of importance in the

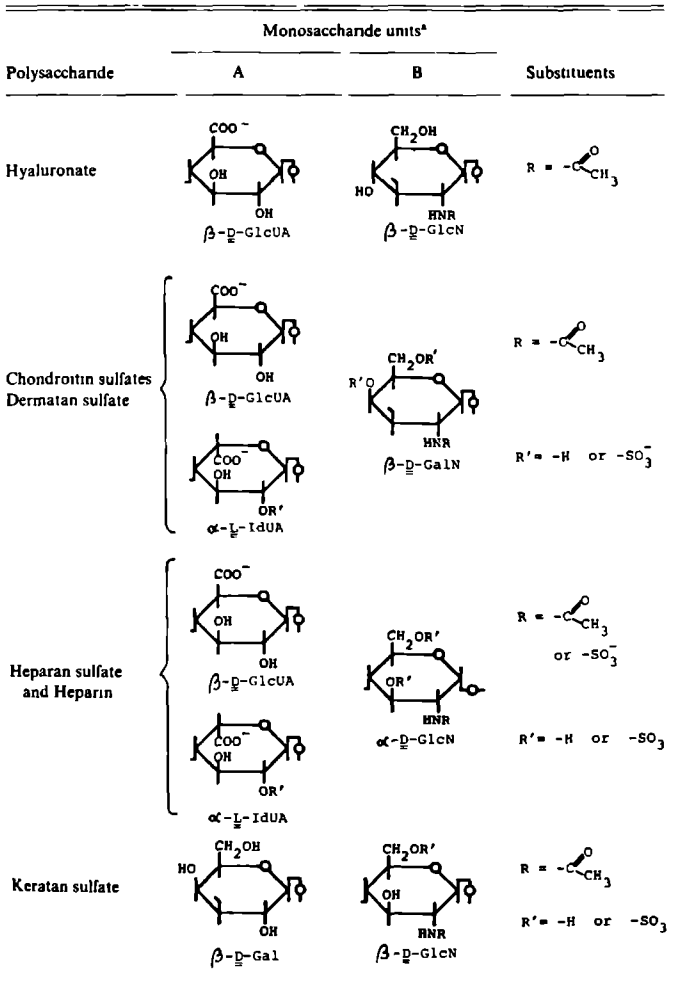

Fig. 6. Structure of glycosaminoglycans. The glycosaminoglycans are linear polymers of alternating A and B monosaccharides units. In dermatan sulphate a part of the uronic acid component is in the form of iduronic acid. GlcUA: glucuronic acid; IdUA: iduronic acid; GlcN: glucosamine; GalN: galactosamine; Gal: galactose. From Höök et al., 1984 in Annual Review of Biochemistry 53, p. 849. Annual Reviews Inc.; with permission.

functioning of proteoglycans (Ts'ao & Eisenstein, 1981; Woodley et al., 1983; Stamatoglou & Keller, 1983; Contri et al., 1985).

Dermatan sulphate proteoglycans are frequently encountered associated with collagen fibrils (Scott et al., 1981; Poole et al., 1986) and may play a role in such phenomena as the structural coherence of collagen fibrils and fibrillogenesis.

The main proteoglycan in the extracellular matrix of cartilage is a huge chondroitin sulphate/keratan sulphate proteoglycan (Handley et al., 1985). These proteoglycans complexe with hyaluronic acid, making up large aggregates (up to 8 µm, Buckwalter et al., 1985). Due to their very high charge density, proteoglycans can bind much water and the aggregates can be regarded as a hydrated gel trapped in a network of collagen fibrils. This organization of the extracellular matrix in cartilage is responsible for its load absorbing properties. When cartilage is compressed, water is forced out of the system, thereby concentrating the proteoglycans and increasing their osmotic acitivity. Upon releasing the force, water flows back into the system.

In the glomerular basement membrane, which separates the circulating blood from the urinary spaces, heparan sulphate proteoglycans act as filter barriers due to their organization as well as their negative charges. Selection of macromolecules depends on their charge as well as their size (Kanwar & Farquhar, 1979).

There are several other functions ascribed to proteoglycans located in the extracellular matrix such as playing a role in mineralization processes (Hascall & Lowther, 1982), and maintenance of the optical transparency in cornea (Hassel et al., 1983). Furthermore, proteoglycans are involved in processes such as cell migration and differentiation (Toole, 1981). It should be noted that although many different functions have been suggested for proteoglycans, hard experimental proof in vivo is difficult to obtain and therefore scarce.

Apart from their presence in the extracellular matrix, proteoglycans can be found at other locations. Heparin proteoglycans are stored inside mast cells; after degranulation and cleavage by an endoglycosidase, the depolymerized products can exert their anticoagulant properties. Heparan sulphate proteoglycans are present as integrated molecules in the cell membrane and are involved in cell recognition and cell attachment phenomena (Hook, 1984). Furthermore, chondroitin sulphate proteoglycans have been found in human platelets (Okayama et al., 1986) and heparan sulphate proteoglycans appear to be present inside nuclei (Gallagher et al., 1986).

The examples of the location and function of proteoglycans given above, are only a grap out of many. Our purpose was merely to indicate that proteoglycans (glycosaminoglycans) are universally present and may play a role in many basic functions of cells and tissues.

Glycosaminoglycans constitute only 0.25-0.5% of the dry weight of the lung parenchyma (Laros et al., 1972; Horwitz & Crystal, 1976; Schmid et al., 1982). All types of glycosaminoglycans are present in the lung, keratan sulphate being restricted to the tracheobronchial cartilage (Mason & Wusteman, 1970). In lung parenchyma, hyaluronic acid, chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin have been demonstrated; chondroitin has not been shown (Schmid et al., 1982). Lung heparan sulphate is known for its variable degree in sulphation (Linker & Hovingh, 1975) and it shows self-associating properties in vitro (Fransson, 1981; Fransson et al., 1981). In human lung, heparin could not be detected biochemically (Schmid et al., 1982). However, because of the presence of mast cells in the lung it is likely that at least some heparin is present. On the contrary, bovine lung is a source of commercial heparin. In bovine and porcine lung, there is a dramatic increase in heparin content during development (Nader et al., 1982).

The different types of glycosaminoglycans are not equally distributed over the various lung components. For instance, in the pleura, dermatan sulphate is the most abundant glycosaminoglycan, while in bronchi it is chondroitin sulphate (Schmid et al., 1982). The amount of glycosaminoglycans also differs from structure to structure. During development there is a change in glycosaminoglycan composition. In general, hyaluronic acid and chondroitin-4-sulphate decrease, while heparan sulphate increases (Horwitz & Crystal, 1975; Radhakrisnamurthy & Berenson, 1980; Schmid et al., 1982).

Human embryonic lung fibroblasts in culture have been shown to synthesize heparan sulphate, dermatan sulphate and chondroitin sulphate proteoglycans; the heparan sulphate proteoglycan was found in the extracellular matrix as well as associated with the cell surface (Vogel & Peterson, 1981; Lories et al., 1986). Human foetal type II epilithelial cells produce hyaluronic acid (Sahu et al., 1980); rat endothelial cells are capable of synthesizing chondroitin sulphate, dermatan sulphate and heparan sulphate/heparin (Cantor et al., 1977).

Ultrastructural studies on proteoglycans (glycosaminoglycans) in lung parenchyma are scarce. Using Ruthenium Red/Triton X-100, heparan sulphate proteoglycans were found in basement membranes, while chondroitin and/or dermatan sulphate proteoglycans were associated with collagen fibrils (Vaccaro & Brody, 1979, 1981). Anionic sites were detected in basement membranes of rat and rabbit lung alveoli using the high iron diamine technique (Sannes, 1984, 1986); using polyethylenimine, Ferrara and coworkers (1985) did the same for human lung

alveoli.

Little is known about the roles proteoglycan and glycosaminoglycans play in the lung. However, their distribution over various lung components, their strategic ultrastructural location and their changes during development suggest they are of crucial importance for the architecture and functioning of the lung.

Emphysema

Emphysema is defined as a condition of the lung characterized by abnormal permanent enlargement of airspaces distal to the terminal brochiole, accompanied by the destruction of their walls, and without obvious fibrosis (National Heart, Lung, and Blood Institute; Workshop on the definition of emphysema, 1985). This definition is an anatomical one, and of limited use for clinical diagnosis. Lung function parameters, related to emphysema, are a decreased forced expiratory volume for one second in the presence of a normal inspiratory volume for one second, an increase in total lung capacity and residual volume, an increase in compliance relative to lung volume and a diminution of diffusing capacity. Chest radiographs are also of importance for diagnosis.

There are two basic approaches in the biochemical and morphological study of emphysema: 1) studyibng pathological human lungs and 2) studying lungs of laboratory animals in which emphysema-like conditions have been artificially induced. Obviously, both approaches have their advantages and disadvantages. The main drawback in animal studies is that one is not sure if the events, occuring in induced emphysema, are the same as in naturally occuring human emphysema. A main advantage is that induced emphysema can be followed from the very beginning in carefully selected animals. Studies on human emphysema, on the other hand, have the main disadvantages that the disease manifests itself clinically when the process is already in an advanced stage. Other complicating factors are the availability of material, coexistence with other diseases, clinical background of the patient, different types of emphysema, etc.

Pathogenesis

The main physiological determinant of emphysema is the loss of elasticity in the lung. The classic discovery of Laurell and Ericksson (1963), that there exists an association between homozygous $alpha_1$-antitrypsine deficiency and

pulmonary emphysema, led to the protease-antiprotease hypothesis. $Alpha_1$-antitrypsine is the principal serum protease inhibitor and a major inhibitor of neutrophil elastase in the human lower respiratory track (Gadek & Crystal, 1982). In short, this theory states that there exists and imbalance between the amount of proteases and antiproteases. Either by an increase of the amount of proteases or a decrease in the amount of antiproteases, the proteases (elastases) would attack and degrade extracellular matrix components (collagen fibrils, elastin, proteoglycans). This would ultimately lead to emphysematous conditions. The use of elastases to provoke panacinar emphysema in animals supports this model (Snider, 1986).

An extension of the protease-antiprotease theory is that there exists and imbalance between oxidants (e.g. hydroxyl radical, H_2O_2, NO_2) and antioxidants (e.g. catalase) resulting in an oxidant burden (Cantin & Crystal, 1985). Oxidants are the result of normal biochemical processes, but may also rise from imflammatory cells (alveolar macrophage, polymorphonuclear leucocytes) and inhaled toxic gasses (cigarette smoke). The antiprotease screen in the lung could be affected by the oxidants, and proteases (e.g. neutrophilic elastase) could attack the connective tissue matrix and lead to alveolar wall destruction.

The protease-antiprotease theory, however, does not readily explain different anatomical types of emphysema (e.g. centriacinar, panacinar) or the male prevalence of the disease in men (Laros & Kuyper, 1978). Furthermore, $alpha_1$-antiproteinase deficiency accounts for less than 1% of all patients (Snider, 1981).

Involvement of proteoglycans and glycosaminoglycans in emphysema

Whatever basic mechanism is responsible for the onset of emphysema, little doubt exists that it is a disturbance in the connective tissue skeleton which ultimately leads to emphysematous conditions. Laros and Kuyper pointed out that proteoglycans may be primary targets in this respect, particularly because of their vulnerability towards proteinases (Laros, 1972; Laros & Kuyper, 1978, 1982). Elastase, used to provoke emphysematous-like conditions, not only degrades elastin but also proteoglycans, collagen and fibronectin (Gadek et al., 1984; Vaccaro et al., 1985). Other enzymes used for inducing emphysema (pronase, papaine), attack the protein core of proteoglycans as well. Since proteoglycans (glycosaminoglycans) determine to large extent the viscosity and cementing properties of the non-fibrillar ground substance, defects in these substances could well lead to a malfunctioning of connective tissue. Overstretching of the

connective tissue framework may be the result which may ultimately lead to emphysematous lesions. Laros et al. (1972) found experimental evidence for their hypothesis. In fresh human lung parenchyma the glucosamine/galactosamine ratio was increased from 0.7 in normal tissue to 3.3 in emphysematous tissue. Because the total amount of glycosaminoglycans, determined as uronic acid, did not change, these data pointed out to a shift in their composition.

Several studies on the involvement of proteoglycans (glycosaminoglycans) in emphysema have been published, the results being contradictionary. An increase of [^{14}C]glucosamine incorporation in hyaluronic acid and heparan sulphate was noted in elastase-induced emphysema (Moczar et al., 1980; Lafuma et al., 1985). The lungs of rats treated with endotoxin, which leads to emphysematous-like conditions, showed an increase of incorporation of [^{35}S]sulphate into total glycosaminoglycans, due to an increase into dermatan sulphate and chondroitin-4-sulphate and a concomitant decrease in heparan sulphate/heparin (Blackwood et al., 1983, 1984). An increase in dermatan sulphate and in total glycosaminoglycan content was found in elastase-treated emphysematous hamster lungs (Karlinsky, 1982), but could not be corroborated (Karlinsky et al., 1983). In human emphysematous lungs, an increase in hyaluronic acid was found (Pecora, 1967), where as Konno and coworkers noted just the opposite (1982). Saltzman et al. (1961) did not find a significant alteration in the glucosamine/galactosamine ratio in human emphysematous lung in contrast to Laros et al. (1972). Differences in experimental setup (time of sampling, organism, inducing agens, analytical methods etc.) probably mainly account for these conflicting results.

Aim of the study

The lung is a highly dynamic organ and undergoes considerable volume changes during the respiratory cycle. The extracellular matrix plays an important role in the maintenance of the structural integrity of the lung. Quantitatively, collagen and elastin are the major elements in the extracellular matrix of the lung. Proteoglycans and glycosaminoglycans are only minor components, but by their strategic location, they may play a keyrole in the functioning of the extracellular matrix.

The research on proteoglycans and glycosaminoglycans of the lung is still in its infancy. Because of the limited amount of these substances in the lung, biochemical studies are difficult. Another complicating factor is the structural complexity of the lung. Many types of tissue structures (small bloodvessels, bronchioli, alveoli etc.) have been completely integrated. Biochemical studies

of these structures proper is not possible. In this respect, ultrastructural studies, in which proteoglycans can be localized and characterized, are of importance. A precise localization may also give a clue as to which roles proteoglycans play in the functioning of normal and pathological lung. The principal aim of this study was to obtain more general knowledge on proteoglycans and glycosaminoglycans of lung parenchyma, histochemically as well as biochemically. Furthermore, the possible involvement of these macromolecules in lung development and emphysema has been investigated.

In Chapter 2, a staining procedure for the ultrastructural localization of proteoglycans in lung alveoli will be described. Using this technique and degradative procedures, the proteoglycans of mouse lung alveoli were characterized (Chapter 3). An attempt to correlate the ultrastructural appearance of proteoglycans of various connective tissues with biochemical data is made in Chapter 4. Using the elaborated staining technique, the proteoglycans of human lung alveoli have been investigated (Chapter 5). One type of the ultrastructurally detected proteoglycans is a dermatan sulphate proteoglycan, which is associated with collagen fibrils. This proteoglycan has been isolated from bovine lung and biochemically analysed (Chapter 6). During the study on proteoglycans from human alveoli, an unusual proteoglycan, characterized by an irregular presence, was detected. The characterization of this proteoglycan is described in Chapter 7. In order to obtain a clue for the function of this proteoglycan, its presence was studied in developing mouse lung (Chapter 8). In Chapter 9, a biochemical and ultrastructural study is presented in which glycosaminoglycans, glycosaminoglycan-degrading enzymes and proteoglycans were investigated in normal and emphysematous human lung parenchyma.

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CHAPTER 2

Staining of proteoglycans in mouse lung alveoli. I. Ultrastructural localization of anionic sites.

TOIN H.M.S.M. VAN KUPPEVELT, JOS G.W. DOMEN, FRANS P.M. CREMERS AND CHARLES M.A. KUYPER

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Staining of proteoglycans in mouse lung alveoli. I. Ultrastructural localization of anionic sites

T. H. M. S. M. VAN KUPPEVELT, J. G. W. DOMEN, F. P. M. CREMERS and C. M. A. KUYPER

Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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Summary

In order to contrast anionic sites in mouse lung alveoli, two staining procedures were applied: (a) staining with Ruthenium Red and Alcian Blue and (b) staining with Cuprolinic Blue in a critical electrolyte concentration method. The Ruthenium Red–Alcian Blue staining procedure revealed electron-dense granules in the alveolar basement membrane. The granules were closely associated with the epithelial cell membrane and continued to stain even when the procedure was carried out at a low pH, indicating the presence of sulphate groups in the granules.

After staining with Cuprolinic Blue, electron-dense filaments, also closely associated with the cell membrane, became visible in the basement membrane of type I epithelial cells. Their length depended on the $MgCl_2$ concentration used during staining. At 0.4 M $MgCl_2$, the length was mostly within the range 100–180 nm. Using a modified Cuprolinic Blue method, the appearance of the filaments closely resembled that of spread proteoglycan monomers with their side-chains condensed. The basement membrane of type II epithelial cells also contained filaments positive towards Cuprolinic Blue; their length, however, was smaller in comparison with those of type I epithelial cells. The filaments lay in one plane and provided the whole alveolus with an almost continuous sheet of anionic sites. Cuprolinic Blue staining also revealed filaments in the basement membrane of the capillary endothelial cells. Furthermore, Cuprolinic Blue-positive filaments (average length about 40 nm) became apparent in close contact with collagen fibrils and separated from each other according to the main banding period of the collagen fibrils (about 60 nm), indicating a specific ultrastructural interaction between these two components. Filaments connecting collagen fibrils with each other were also detected.

Introduction

Proteoglycans are macromolecules consisting of a protein component and a polysaccharide component, the latter represented by the so-called glycosaminoglycans. The glycosaminoglycans (except keratan sulphate) are long chains of repeating disaccharides, consisting of a hexosamine and an uronic acid residue. The current model for proteoglycans is a protein core to which the glycosaminoglycans are covalently

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attached. Using isolated proteoglycans, such a model can be visualized in the electron microscope (Rosenberg *et al.*, 1974; Thyberg *et al.*, 1975). However, in tissues, such a structure has not yet been demonstrated.

Proteoglycans are thought to have important functions in tissues, although their contribution to the tissue dry weight is mostly quite small; for example, in lung, the proteoglycans constitute only 1% of the dry weight, about half of that being occupied by glycosaminoglycans (Horwitz *et al.*, 1976). The principal functions of proteoglycans are the following: acting as a filter barrier (Kanwar *et al.*, 1980), influencing morphogenesis (Silberstein & Daniel, 1982; Lash & Vasan, 1978), playing a role in cell recognition (Fransson *et al.*, 1981), and absorbing compressive load (Hascall & Hascall, 1981). Because of this participation of proteoglycans in a variety of tissue functions, it follows that they may also be involved in several diseases. For instance, it has been shown from biochemical studies that in pulmonary emphysema (Laros *et al.*, 1972; Moczar *et al.*, 1980) and pulmonary fibrosis (Nagai *et al.*, 1977; Motomiya *et al.*, 1975), changes occur in the glycosaminoglycan composition of the lung.

However, it is difficult to interpret which structural element is responsible for the biochemical data from lung blocks. This is due to the structural complexity of the lung, in which many types of tissue components (blood vessels, bronchioli, alveoli, etc.) are completely integrated, making it impossible to analyse biochemically the proteoglycan (glycosaminoglycan) content of lung alveoli proper. In contrast, histochemical staining procedures provide useful information regarding the localization and composition of proteoglycans and hence, they can supplement biochemical investigations. Several dyes have been used for demonstrating proteoglycans at the ultrastructural level, for example, Alcian Blue (Goldberg *et al.*, 1978), Ruthenium Red (Myers *et al.*, 1973; Gordon & Bernfield, 1980), Toluidine Blue (Shepard & Mitchell, 1976), Acridine Orange (Lapis & Timár, 1980) and cationized ferritin (Kanwar & Farquhar, 1979). This staining of proteoglycans is presumably based on the electrostatic attraction between the positive dye and the polyanionic glycosaminoglycans (Scott, 1973). However, due to their collapse during the preparation of tissue, the proteoglycans appear as electron-dense granules rather than extended, open structures. In order to contrast proteoglycans, Scott (1980) introduced the phthalocyanin-like dye, Cuprolinic Blue, using it according to the critical electrolyte concentration method.

In this paper, we report the application of this staining procedure, as well as a combination of Alcian Blue and Ruthenium Red, for identifying anionic sites in lung alveoli.

Materials and methods

Three-month-old mice (K-strain, inbred) were sacrificed and the lungs removed. Small lung blocks (1 mm × 1 mm × 1 mm) were placed in fixative and, in order to obtain thorough penetration of the fixative, degassed.

Ruthenium Red–Alcian Blue staining

Lung blocks were fixed (4 h, room temperature) in 0.1 M sodium cacodylate buffer (pH 7.2),

containing 2.5% glutaraldehyde, 0.2 or 2.0% Ruthenium Red (Luft, 1971, Aldrich-Europe, Beerse, Belgium) and 0.2 or 2.0% Alcian Blue (8GX, Serva, Heidelberg, West Germany). In this study, only a combination of Ruthenium Red and Alcian Blue resulted in a good and reproducible contrast of anionic structures. After washing in 0.1 M cacodylate buffer the tissue blocks were left overnight (4° C) in the same solution as the fixative, but without glutaraldehyde. Postfixation (4 h, room temperature) took place in 0.05 M cacodylate buffer (pH 7.2) containing 1% OsO_4, 0.2 or 2.0% Ruthenium Red and 0.2 or 2.0% Alcian Blue, followed by dehydration in ascending concentrations of ethanol.

In one case the cacodylate buffer was replaced by veronal-acetate–HCl buffer (pH 2.6) using the same molarities.

Cuprolinic Blue staining

Lung blocks were fixed in 0.025 M sodium acetate buffer (pH 5.6) containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue (BDH Ltd, Poole, U K.) and 0.1–0.4 M $MgCl_2$. After fixation overnight (room temperature) the specimens were washed three times (for a total time of 30 min) in the same solution, but without Cuprolinic Blue. After washing three times in 1% aqueous Na_2WO_4, the specimens were dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% sodium tungstate unless otherwise stated.

In addition, a modified Cuprolinic Blue staining procedure was applied; the specimen was allowed to stand for 1 h in the sodium acetate buffer containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue and 0.2 M $MgCl_2$, then washed twice, each for 10 min, in the same solution without Cuprolinic Blue. Next the lung blocks were placed for 2 h in buffer containing 2.5% glutaraldehyde, 0.5% sodium tungstate and 0.2 M $MgCl_2$, washed twice for 10 min periods in the same solution without tungstate and allowed to stand for 2 h in buffer containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue, 0 5% sodium sulphate and 0 2 M $MgCl_2$, followed by dehydration.

After dehydration, the specimens were embedded in Epon (Luft, 1961) following standard procedures. Ultrathin sections were collected on formvar-coated grids. Occasional sections were poststained with uranyl acetate and lead citrate (Reynolds, 1963), or with 0.5% sodium tungstate in a 50% (v/v) ethanol–water mixture. Sections were examined in a Philips 201 electron microscope at 60 kV.

Results

Ruthenium Red–Alcian Blue staining

The tissue layer which separates the blood from the alveolar airspace is called the blood–air barrier (Fig. 1) and is formed by an epithelial cell layer, an alveolar basement membrane and an endothelial cell layer. The epithelium and its alveolar basement membrane enclose the whole alveolus.

After staining with Ruthenium Red–Alcian Blue, electron-dense granules appear in the alveolar basement membrane (Fig. 2a). They are closely associated with the epithelial cell membrane and mostly separated from each other by a distance of about 60 nm (Fig. 2a, arrows). When the basement membrane is cut tangentially a field of electron-dense granules becomes visible (Fig. 2a), the granules lying in a quasi-regular pattern, similar to that observed previously for the renal glomerular basement membrane (Kanwar & Farquhar, 1979). When the pH of the fixative is lowered (pH 2.6),

the granules are still present (Fig 2b), indicating the presence of sulphate groups, since carboxylic groups are hardly ionized at such a pH No electron-dense granules were seen associated with collagen fibrils.

When only Alcian Blue is present during glutaraldehyde fixation and only Ruthenium Red is present during postfixation with OsO_4, no electron-dense granules become apparent Without Ruthenium Red–Alcian Blue no granules are visible.

Cuprolinic Blue staining

Staining with Cuprolinic Blue reveals electron-dense filaments (Fig 3), located in the alveolar basement membrane of type I epithelial cells and closely associated with their

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Fig. 1. Blood–air barrier, which separates the blood from the alveolar airspace It consists of an epithelial cell layer (ep), an alveolar basement membrane (abm) and an endothelial cell layer (en) e, erythrocyte, a, alveolus, cap, capillary Glutaraldehyde–OsO_4 fixation, uranyl acetate + lead citrate

Fig. 2. Blood–air barrier after staining with Ruthenium Red–Alcian Blue, uranyl acetate + lead citrate (a) Ruthenium Red–Alcian Blue staining reveals electron-dense granules, lying in the alveolar basement membrane and closely associated with the epithelium Most are separated from each other by a distance of 60 nm (arrows), when the alveolar basement membrane is cut tangentially a field of granules becomes apparent (b) Ruthenium Red–Alcian Blue staining at low pH The alveolar basement membrane granules continue to stain

Fig 3 Blood–air barrier after Cuprolinic Blue staining (0 3 M $MgCl_2$) In the basement membrane of type I epithelial cells, electron-dense filaments (bmf I) become visible When the alveolar basement membrane is cut perpendicularly the filaments can be seen linearly aligned Sodium tungstate was used only in the poststaining step

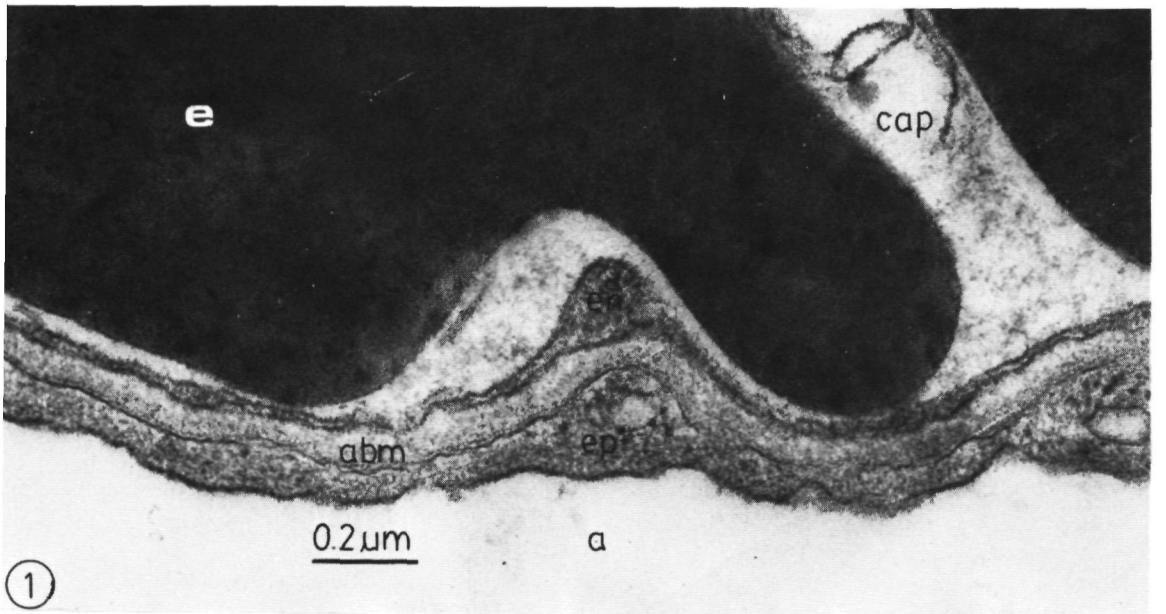
Fig. 4. Tangentially sectioned alveolar basement after Cuprolinic Blue staining (0 4 M $MgCl_2$) Sodium tungstate was used during the wash and dehydration steps the bmf I are stained more heavily compared with those in Fig 3 The transition of the perpendicularly sectioned alveolar basement membrane to the tangentially sectioned membrane is indicated In the tangentially cut alveolar basement membrane, fields of basement membrane filaments become apparent, the length of the basement membrane filaments is mostly within the range of 100–180 nm

Fig. 5. Alveolar basement membrane after Cuprolinic Blue staining at 0 15 M $MgCl_2$ The average length of the basement membrane filaments I is approximately 50 nm

Fig. 6. Tangentially sectioned alveolar basement membrane after applying the modified Cuprolinic Blue staining procedure (see Materials and methods) The basement membrane filaments I appear to be thicker and contain electron-dense dots (arrows) Inset occasional projection arising from the filaments are visible (arrows)

Fig. 7. Alveolar basement membrane of type II epithelial cells (ect II) after Cuprolinic Blue staining (0 3 M $MgCl_2$) Electron-dense epithelium-associated filaments (bmf II) become apparent in the alveolar basement filament The appearance of multilamellar bodies (mb) indicates that phospholipids are washed out, as a result of the procedure used Because of the specific interaction of Cuprolinic Blue with nucleic acids ribosomes (rib) also stain

Fig. 8. Capillary basement membrane (cbm) after Cuprolinic Blue staining (0 2 M $MgCl_2$) Electron dense filaments (bmf III), more scattered in comparison with those located in the alveolar basement membrane, become visible in the capillary basement membrane



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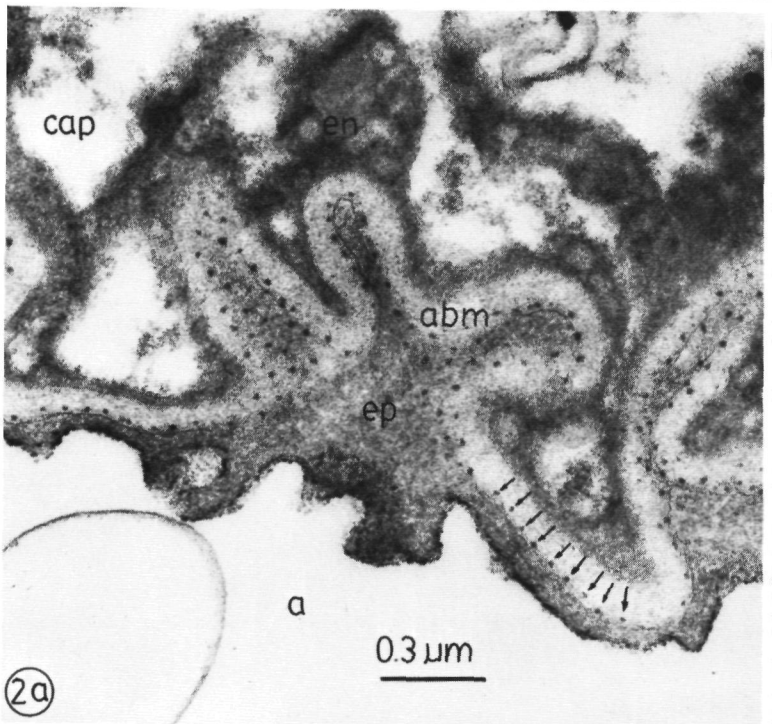

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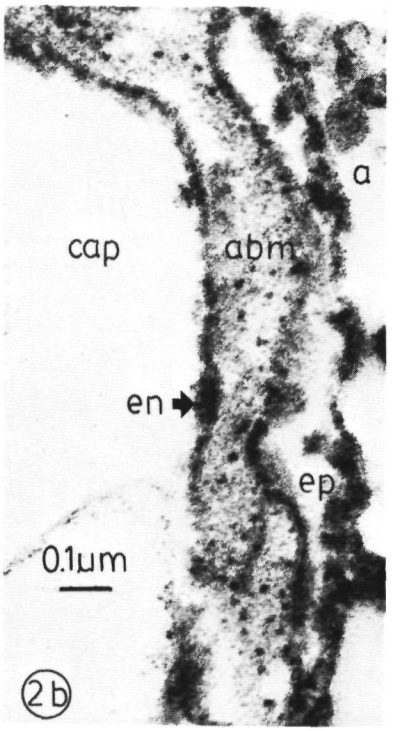

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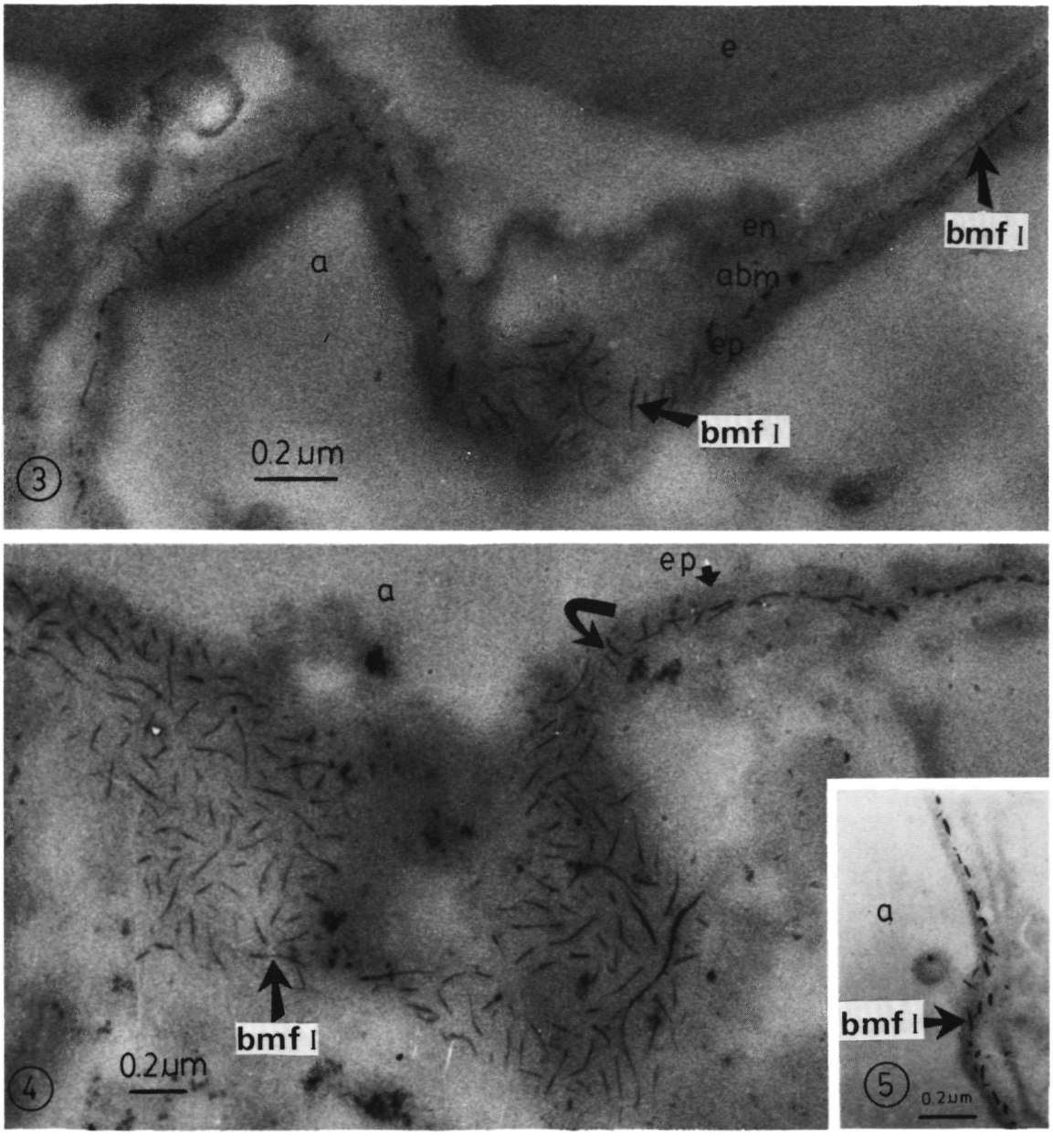
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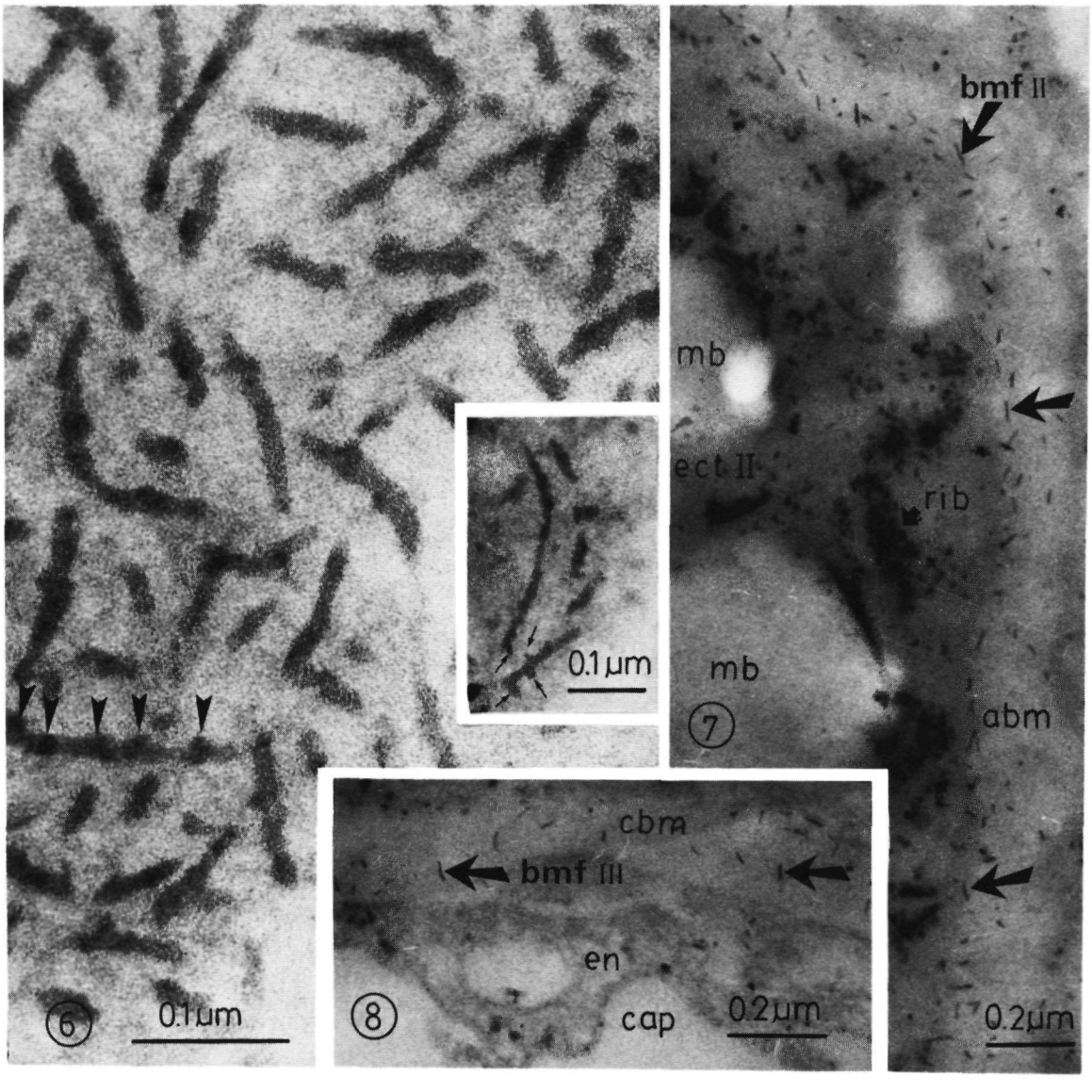
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cell membrane. These Cuprolinic Blue-positive filaments appear to lie in one plane, thus providing the alveolus with an almost continuous sheet of anionic sites. When a filament is cut perpendicularly, it becomes visible as an electron-dense dot (Fig. 3). In grazing sections of the alveolar basement membrane, fields of these filaments become apparent (Fig. 4). Staining with sodium tungstate whilst being washed and dehydrated (Fig. 4) results in filaments of greater electron density becoming evident than when sodium tungstate is applied only in the poststaining step proper (Fig. 3). The length of these basement membrane filaments (bmf I) depends on the $MgCl_2$ concentration used during staining; for instance, at 0.15 M $MgCl_2$ their average length is about 50 nm (Fig. 5), while at 0.4 M $MgCl_2$ their length is mostly within the range of 100–180 nm (Fig. 4). Very large filaments (Fig. 4) are considered to be fusions of filaments. The filaments at a lower $MgCl_2$ concentration appear to be thicker and are more intensely stained. When applying the modified Cuprolinic Blue staining procedure (see Materials and methods) the filaments appear to be thicker (Fig. 6); furthermore, rounded electron-dense dots can be detected on the filaments and sometimes projections arising from the filaments are visible (Fig. 6, inset).

The filaments thus far described are located in the alveolar basement membrane associated with type I epithelial cells. Type II epithelial cells, which produce phospholipids of the surfactant system, are also associated with an alveolar basement membrane which provides only a minor contribution to the total amount of alveolar basement membrane. Filaments, which are smaller than those associated with type I cells, can be detected in this basement membrane (Fig. 7, bmf II). Furthermore, the capillary basement membrane, associated with the endothelium, also contains Cuprolinic Blue-positive structures (Fig. 8, bmf III) but they are more scattered in comparison with those located in the alveolar basement membrane. This is independent of the $MgCl_2$ concentration used.

In addition to the basement membrane associated filaments, collagen-associated filaments can also be detected in the alveolar wall after Cuprolinic Blue staining (Fig. 9). These filaments are about 40 nm long, their length being much less dependent on the $MgCl_2$ concentration used, and are separated from each other according to the main banding period of fibrils (±60 nm). In some cases these filaments connect collagen fibrils

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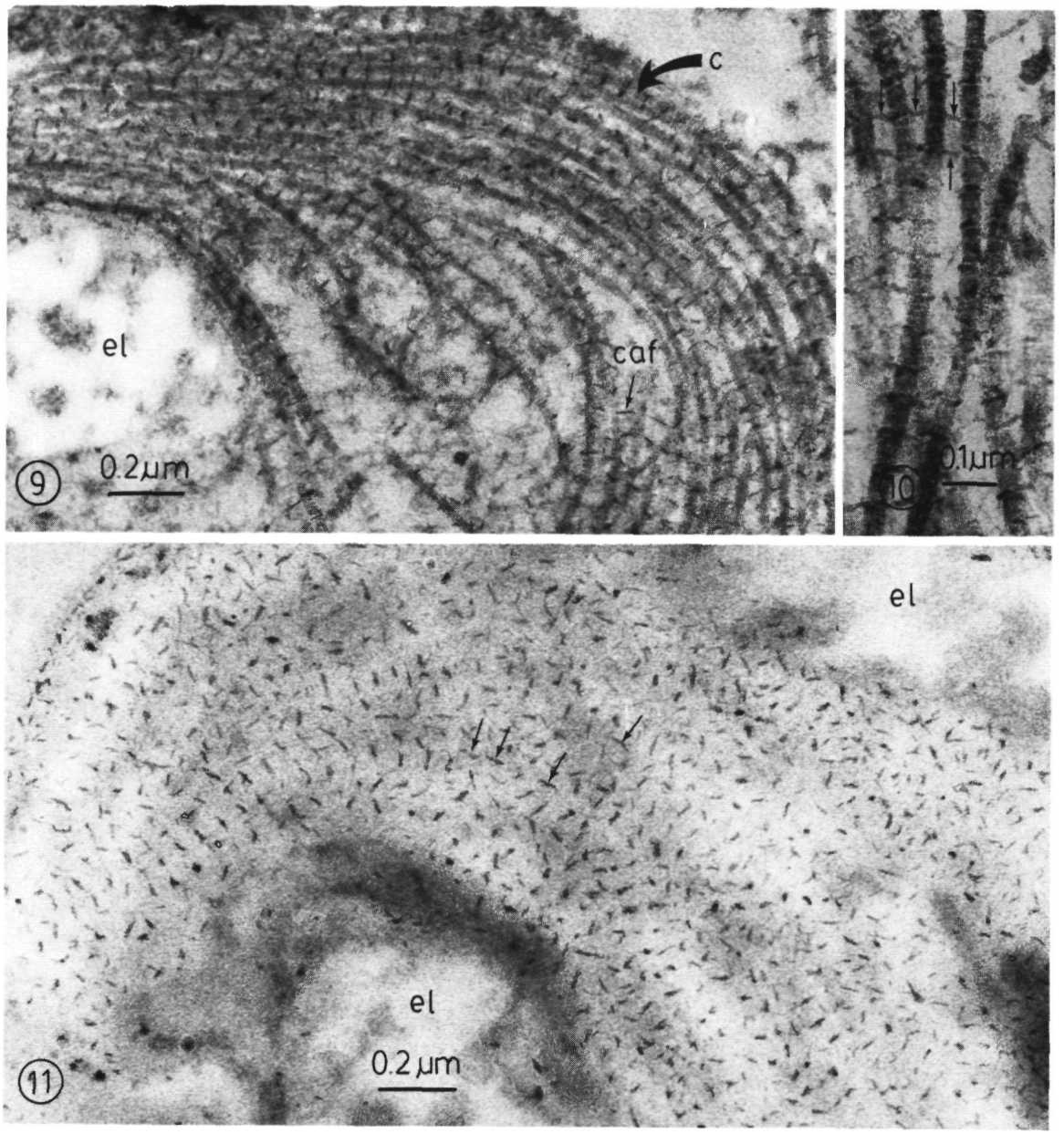
Fig. 9. Collagen fibrils after Cuprolinic Blue staining (0.2 M $MgCl_2$), uranyl acetate + lead citrate. Collagen associated filaments (caf) become apparent; their distance from each other follows the main banding period of the collagen fibrils (about 60 nm). c, collagen fibril; el, elastin.

Fig. 10. Collagen fibrils after Cuprolinic Blue staining (0.2 M $MgCl_2$), uranyl acetate + lead citrate. The collagen fibrils are connected to each other by Cuprolinic Blue-positive filaments (arrows).

Fig. 11. Collagen associated filaments (0.1 M $MgCl_2$). When poststaining with uranyl acetate and lead citrate is omitted, the collagen fibrils cannot be detected; the collagen associated filaments (caf), however, are still present and reflect the position of the collagen fibrils; besides numerous collagen associated filaments which lie perpendicular to the fibril axis, some can be detected lying in a parallel fashion (arrows).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with each other (Fig. 10). Although most filaments are lying perpendicular to the fibril axis, some of them lie parallel (Fig. 11). When poststaining with uranyl acetate and lead citrate is omitted, the collagen fibrils can no longer be detected; the collagen-associated filaments, however, remain clearly visible (Fig. 11). From the pattern of the filaments, it is possible to deduce the position of the collagen fibrils.

Without Cuprolinic Blue, no filaments are visible. Intracellularly the nucleus and the ribosomes stain (Fig. 7), because of the specific interaction of Cuprolinic Blue with nucleic acids (Scott, 1972).

Discussion

Both the Ruthenium Red–Alcian Blue and the Cuprolinic Blue staining procedures contrast particles in the basement membrane, but only Cuprolinic Blue can also clearly contrast filaments associated with collagen fibrils. Vaccaro and Brody (1979), using Ruthenium Red for identifying anionic sites in lung alveoli, contrasted granules in the alveolar basement membrane as well as granules associated with collagen fibrils, but they had to use the non-ionic detergent Triton X-100 in order to obtain good penetration of the dye. The absence of Ruthenium Red–Alcian Blue-positive, collagen-associated granules might, therefore, be caused by a poor penetration of these dyes. After Cuprolinic Blue staining, however, filaments associated with collagen fibrils can be detected easily. As Triton X-100 severely damages the lung ultrastructure, Cuprolinic Blue staining is preferable for contrasting anionic sites in lung aveoli.

Clearly, these two staining procedures contrast the same anionic sites in the alveolar basement membrane but there is a striking difference in their appearance. The Cuprolinic Blue-positive structures are visualized as filaments, while the Ruthenium Red–Alcian Blue-positive structures appear as granules. Furthermore, the length of the Cuprolinic Blue-positive filaments depends on the $MgCl_2$ concentration used during staining. Addition of $MgCl_2$, therefore, seems important for keeping the anionic structures in a more extended form, although a specific interaction of Cuprolinic Blue might also contribute to the difference in appearance compared to that given by the Ruthenium Red–Alcian Blue method.

It is also possible to derive information concerning the nature of the anionic sites. The Ruthenium Red–Alcian Blue-positive granules continue to stain even at pH 2.6, at which level the carboxylic acid groups are poorly ionized, in contrast with sulphate groups. This indicates that sulphate groups are probably present. The Cuprolinic Blue staining is performed in the presence of $MgCl_2$. It has been shown (Scott, 1972) that, due to the competitive action of Mg^{2+} ions, cationic dyes are easily displaced from carboxylic groups at a relatively low concentration of $MgCl_2$ (about 0.05 M), but sulphate groups need considerably higher concentrations of $MgCl_2$ for displacement. Hence, it is likely that the Ruthenium Red–Alcian Blue and Cuprolinic Blue-positive structures contain sulphate groups, indicating that these anionic sites contain sulphated glycosaminoglycans. Furthermore, after applying the modified Cuprolinic Blue staining procedure, the appearance of the filaments closely resembles that of spread

proteoglycan monomers with the glycosaminoglycan chains condensed (Hascall, 1980, Buckwalter & Rosenberg, 1982, Buckwalter *et al.*, 1982) Therefore, the filaments probably represent proteoglycans. This is in agreement with the study of Hascall (1980), who showed that the Ruthenium Red-positive granules, as observed in rat chondrosarcoma, correspond to the condensed form of proteoglycan monomers This also corresponds with the study of Scott (1980), who considered the Cuprolinic Blue-positive filaments, as observed in rat tail tendon, to be proteoglycans, the filamentous structure would be caused by the collapse of the glycosaminoglycan chains on the protein core of the proteoglycan The Cuprolinic Blue staining procedure, however, still gives a better representation of the proteoglycans than, for instance, Ruthenium Red

In the alveolar basement membrane, the Cuprolinic Blue-positive filaments lie in one plane and are parallel to the epithelial plasma membrane. They are arranged in such a way as to almost enclose the alveolus completely Hence, it seems probable that the alveolar wall contains a continuous sheet of anionic sites.

The Cuprolinic Blue-positive filaments associated with the collagen fibrils are separated from each other according to the main banding period of the collagen fibrils (± 60 nm), indicating a specific interaction between these two components However, this distance is probably somewhat larger *in vivo*, since it is known that glutaraldehyde fixation results in a shrinkage of the collagen fibril (Meek, 1981)

Ultrastructural association of cationic dye-positive structures and collagen fibrils is well documented in a variety of tissues other than lung alveoli, for instance, predentine (Nygren *et al* , 1976), arteries (Wight & Ross, 1975), and embryonic cornea (Trelstad *et al.*, 1974). In these tissues, stained with either Ruthenium Red or Alcian Blue, the anionic granules were mostly observed to be next to the collagen fibrils However, Scott (1980), using Cuprolinic Blue and a critical electrolyte concentration method, noted that in the tendon, electron-dense filaments were present around the collagen fibrils, and there were also filaments running parallel to the fibril axis. In our study we found few filaments running parallel to the axis in comparison to those running perpendicularly. Futhermore, filaments connecting collagen fibrils to each other could be detected. Hence, it is possible that collagen fibrils in the lung alveoli are enclosed by a network of these anionic filaments and in such a way provide structure coherence.

In conclusion, there are several indications pointing to the proteoglycan nature of the Cuprolinic Blue-positive filaments described here, the difference in length and localization of the various filaments suggests a difference in chemical composition of these anionic structures. In the accompanying paper, the characteristics of these filaments are described further

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CHAPTER 3

Staining of proteoglycans in mouse lung alveoli. II. Characterization of the Cuprolinic Blue-positive, anionic sites.

TOIN H.M.S.M. VAN KUPPEVELT, FRANS P.M.CREMERS, JOS G.W. DOMEN AND CHARLES M.A. KUYPER

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Staining of proteoglycans in mouse lung alveoli. II. Characterization of the Cuprolinic Blue-positive, anionic sites

T. H. M. S. M. VAN KUPPEVELT, F. P. M. CREMERS, J. G. W. DOMEN and C. M. A. KUYPER

Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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Summary

The nature of Cuprolinic Blue-positive anionic filaments in mouse lung alveoli has been characterized. The contrast of filaments in the alveolar basement membrane of type I epithelial cells was lost on treatment with nitrous acid and pronase (without prefixation). In contrast, neither neuraminidase, chondroitinase ABC or AC, nor *Streptomyces* hyaluronidase had any effect. Treatment with pronase (after prefixation) and 2.0 M $MgCl_2$ (after prefixation) also had no effect, indicating that the filaments are heparan sulphate proteoglycans. The filaments in the alveolar basement membrane of type II epithelial cells and in the capillary basement membrane of the endothelial cells were also nitrous acid sensitive, but chondroitinase ABC-insensitive. A model in which the whole alveolus contains a single layer of heparan sulphate-containing proteoglycan monomers is proposed. Furthermore, the collagen fibril associated filaments remained unaffected after treatment with nitrous acid, neuraminidase or *Streptomyces* hyaluronidase, or after digestion with pronase (after prefixation) and treatment with 2.0 M $MgCl_2$ (after prefixation). These filaments, however, could no longer be detected when digestion with chondroitinase ABC or pronase (without prefixation) was applied; chondroitinase AC treatment clearly affected the filaments, although they still were visible. These results indicate that the filaments are dermatan sulphate-containing proteoglycans. Some functional aspects of the proteoglycans are discussed.

Introduction

Several types of glycosaminoglycans exist, varying in either the hexosamine component, the uronic acid component or the number and localization of sulphate groups. The hexosamine constituent in dermatan sulphate and chondroitin 4-/6-sulphate is galactosamine, whereas in hyaluronic acid, heparan sulphate and heparin it is glucosamine. Glucuronic acid is the uronic acid constituent in hyaluronic acid and chondroitin sulphate, but a considerable part of the uronic acid in dermatan sulphate,

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heparan sulphate and heparin is iduronic acid. Keratan sulphate is the only glycosaminoglycan without an uronic acid component. The presence of hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparan sulphate and heparin has been demonstrated in lung (Horwitz & Crystal, 1975; Sampson *et al.*, 1979; Schmid *et al.*, 1982), keratan sulphate being present only in the tracheobronchial cartilage (Horwitz *et al.*, 1976).

Specific roles of the various pulmonary glycosaminoglycans can be assumed from observations such as the changing of glycosaminoglycan constitution during lung development (Horwitz & Crystal, 1975, Schmid *et al.*, 1982) and the distribution of different glycosaminoglycans in various lung structures (Radhakrishnamurthy *et al.*, 1980; Schmid *et al.*, 1982).

A correlation of the type of glycosaminoglycan or proteoglycan with its precise ultrastructural localization is of great importance, since it can give information about the function of the specific glycosaminoglycan or proteoglycan. This is a prerequisite when studying a disease in which glycosaminoglycans or proteoglycans might be involved. In this paper the Cuprolinic Blue-positive anionic sites, as described in the previous paper, are characterized further, mainly by the use of enzymes.

Materials and methods

Male mouse lung blocks were obtained and degassed as described in the accompanying paper.

Enzyme digestion

In order to determine the nature of the Cuprolinic Blue-positive filaments, various enzymes were used according to the conditions given in Table 1. All the enzyme solutions (except pronase) contained 5 mM benzamidine HCl (Aldrich-Europe, Beerse, Belgium) and 0 1 M 6-amino-*n*-caproic acid (Janssen Chimica, Beerse, Belgium) (Maeda *et al.*, 1981) to avoid damage to the tissue by proteolytic enzymes, which are either from the tissue itself or are contaminants of the enzymes employed. Glycosaminoglycan-degrading enzymes and nitrous acid were tested for their specificity using cellulose acetate electrophoresis (see below). Unless otherwise stated, the tissue was degassed (on ice) in the enzyme solution and placed in a waterbath at 37° C As controls, the same solutions were used without the enzyme.

In one case, two enzymes (chondroitinase ABC and *Streptomyces* hyaluronidase) were used. First, chondroitinase ABC was employed under the conditions shown in Table 1 The tissue was then washed (10 min, 0° C) in the solution containing protease inhibitors and no enzyme, washed again (10 min, 0° C) in sodium chloride–acetate buffer containing protease inhibitors and finally, subjected to hyaluronidase digestion (Table 1).

Pronase treatment was carried out either directly or after prefixation of the tissue; no protease inhibitors were used. Prefixation (2 h, 4° C) was carried out in 0.025 M sodium acetate buffer (pH 5.6) containing 2.5% glutaraldehyde, and after washing twice (10 min, 4° C) in Tris buffer (enriched, Table 1), the tissue was placed in the pronase solution (Table 1).

Nitrous acid treatment

In order to degrade specifically heparan sulphate and heparin, nitrous acid was used (Kosher & Searls, 1973): 5% $NaNO_2$ and 33% acetic acid (1:1 v/v) were mixed and allowed to stand for 90

Table 1. Enzymes used and conditions employed for characterization of the Cuprolinic Blue-positive, anionic sites

Enzyme	*Source*	*Concentration + buffer*	*pH*	*Temperature*	*Time* (min)	*Reference**
Chondroitinase ABC	*Proteus vulgaris* (Sigma)	1U/ml enriched Tris buffer†	8.0	37 °C	45	Vaccaro & Brody, 1979, Ausprunk, 1981
Chondroitinase AC	*Arthrobacter aurescens* (Sigma)	1U/ml enriched Tris buffer	8.0	37° C	45	—
Hyaluronidase	*Streptomyces* (Calbiochem)	5 T R U ‡/ml NaCl–acetate buffer§	5.4	37° C	45	Kanwar *et al* , 1980, Vaccaro & Brody, 1979
Neuraminidase	*Clostridium perfringens* (Sigma)	1U/ml NaCl–acetate buffer	5.4	37° C	45	Kanwar & Farquhar, 1979.
Pronase	*Streptomyces griseus* (Calbiochem)	15U/ml enriched Tris buffer	8.0	37° C	45	—

*The enzyme conditions have been adopted from the references given here, occasionally some modifications have been introduced
†0.25 M Tris, 0.18 M NaCl, 0.05% BSA
‡T R U = Turbidity Reducing Units
§0.1 M sodium acetate, 0.15 M NaCl

min at room temperature, after which 5 mM benzamidine HCl and 0 1 M 6-amino-*n*-caproic acid were added The tissue was first subjected to prefixation with glutaraldehyde (see Pronase digestion) and after rinsing twice in 0 2 M sodium acetate containing protease inhibitors, the lung blocks were incubated in nitrous acid for 90 min at room temperature As a control a 0 2 M sodium acetate solution, also containing protease inhibitors, was used with the same pH as the nitrous acid solution

$MgCl_2$ treatment

In order to investigate the nature of the interaction between the glycosaminoglycans and proteins the tissue was subjected to treatment with $MgCl_2$ Lung blocks were allowed to stand for 2 h (room temperature) in 0 025 M sodium acetate buffer, pH 5 6, containing protease inhibitors and 0 2 or 2 0 M $MgCl_2$ This was followed by three 10 min washes at room temperature in the same solution, one wash (10 min, room temperature) in buffer without $MgCl_2$ and finally a rinse (10 min, room temperature) in buffer containing 0 2 M $MgCl_2$ in order to make the tissue suitable for staining and fixation Staining and fixation are also performed in the presence of 0 2 M $MgCl_2$ (see below) In one instance, the 2 0 M $MgCl_2$ treatment was preceded by prefixation followed by one wash (10 min, room temperature) in the sodium acetate buffer containing protease inhibitors and 2 0 M $MgCl_2$

Fixation and Cuprolinic Blue staining

After enzymic or nitrous acid treatment, the specimen was washed once (10 min, 0° C) in the control solution and after being washed once again (10 min, 0° C) in 0 025 M sodium acetate buffer (pH 5 6) containing protease inhibitors, the lung blocks were fixed and stained (overnight, room temperature) in 0 025 M sodium acetate buffer containing 2 5% glutaraldehyde, 0 2% Cuprolinic Blue and 0 2 M $MgCl_2$ After fixation and staining the material was washed three times in the fixative solution without Cuprolinic Blue, three times in 1% aqueous sodium tungstate and dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% sodium tungstate Lastly, the lung blocks were embedded in Epon and ultrathin sections were examined with a Philips 201 electron microscope

Cellulose acetate electrophoresis

In order to test the specificity of the glycosaminoglycan degrading enzymes and nitrous acid a mixture of pure glycosaminoglycans was subjected to treatment with enzyme or nitrous acid, after which electrophoresis was performed in cellulose acetate strips using copper acetate (Stefanovich & Gore, 1967) as buffer

Results

As described in the previous paper, four kinds of electron-dense filaments became visible in lung alveoli after Cuprolinic Blue staining one (basement membrane filaments I) in the alveolar basement membrane of type I epithelial cells was closely associated with the plasma membrane, one (basement membrane filaments II) associated with the alveolar basement membrane of type II epithelial cells, another (basement membrane filaments III) was located in the basement membrane of the capillary endothelium, and the last type associated with collagen fibrils Due to the specificity of the staining procedure the Cuprolinic Blue-positive filaments are sometimes the only structures clearly visible The electron density of collagen fibrils is variable, poststaining with lead

Table 2. Effects of various treatments on the basement membrane filaments I (bmf I) and the collagen associated filaments (caf)

Treatment	*Substrate*	*Effect on bmf I*	*Effect on collagen associated filaments*
Neuraminidase	sialoglycoproteins	–	–
Streptomyces hyaluronidase	hyaluronic acid	–	–
Chondroitinase ABC	chondroitin sulphate, dermatan sulphate	–	+
Chondroitinase AC	hyaluronic acid, chondroitin sulphate, dermatan sulphate only where it contains a glucuronic residue	–	±
Chondroitinase ABC + *Streptomyces* hyaluronidase	hyaluronic acid, chondroitin sulphate, dermatan sulphate	–	+
Nitrous acid	heparan sulphate, heparin	+	–
Pronase (after prefixation)	proteins	–	–
Pronase (without prefixation)	proteins	+	+
2 0 M $MgCl_2$ (after prefixation)	ionic bonds	–	–
2 0 M $MgCl_2$ (without prefixation)	ionic bonds	–	+

– no effect
+, filaments no longer visible,
±, stainability of filaments reduced

citrate, with or without uranyl acetate, however, is not always desirable since it may obscure the Cuprolinic Blue-positive filaments The length of the filaments, as described in the previous paper, is sometimes diminished, possibly as a result of the pretreatment of the tissue

Neuraminidase treatment, which splits off sialic acid from glycoproteins, has no effect on the stainability of the basement membrane filaments type I (Fig 1a) or the collagen associated filaments (Fig 1b) Digestion with *Streptomyces* hyaluronidase, which degrades hyaluronic acid specifically, also has no effect On the other hand, treatment with chondroitinase ABC, which digests chondroitin sulphate and dermatan sulphate, results in the complete disappearance of the stainability of the collagen associated filaments (Fig 2b), the basement membrane filaments I remaining unaffected (Fig 2b). In the control (Fig 2a) both kinds of filaments are clearly visible. When lung tissue is treated with chondroitinase AC, which breaks down hyaluronic acid and chondroitin sulphate, but breaks down dermatan sulphate only where it contains a glucuronic acid residue, a complete removal of the staining of the collagen associated filaments is not obtained, although these filaments are clearly affected by the enzyme treatment (Fig. 2c). Occasionally, however, this effect is less pronounced. The basement membrane

filaments I, again, remain unaltered. Using a combination of *Streptomyces* hyaluronidase and chondroitinase ABC all glycosaminoglycans are degraded, with the exception of heparan sulpate and heparin (and keratan sulphate, which is not present, however, in lung alveoli). After treatment with this combination of enzymes, basement membrane filaments type I remain unaffected (Fig. 3a), the collagen associated filaments are no longer detectable (Fig. 3b). Nitrous acid treatment, which causes degradation of heparan sulphate and heparin, has an opposite effect: the basement membrane filaments I are no longer stained (Fig. 4a), while the collagen associated filaments are not affected (Fig. 4b).

After pronase digestion, without prefixation, the basement membrane filaments I and collagen associated filaments (Fig. 5b) can no longer be detected. When, however, prefixation with glutaraldehyde (which fixes proteins by cross-linking) (Geyer, 1973; Cheung & Nimni, 1982) is applied, the pronase has no influence on the staining (Fig. 5a). Furthermore, treatment of the tissue with 2.0 M $MgCl_2$ (which will attack ionic bonds) after prefixation, has no effect on the filaments (Fig. 6a,b). After treatment with 2.0 M $MgCl_2$ without prefixation, however, the basement membrane filaments I remain stained (Fig. 7a), but the collagen associated filaments are no longer visible (Fig. 7b); when the

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Fig. 1. Part of alveolar wall after neuraminidase treatment. (a) The Cuprolinic Blue-positive filaments (bmf I) in the basement membrane of type I epithelial cells remain unaffected. (b) The Cuprolinic Blue-positive, collagen fibril associated filaments (caf) also are not affected a, alveolus; c, collagen fibril.

Fig. 2. Part of alveolar wall after chondroitinase ABC or AC treatment. (a) Control. the basement membrane filaments type I and collagen associated filaments are clearly visible. (b) Chondroitinase ABC digestion: the basement membrane filaments type I remain unaffected, while the collagen associated filaments are not detectable anymore. (c) Chondroitinase AC: the collagen associated filaments are clearly affected but remain visible. el, elastin.

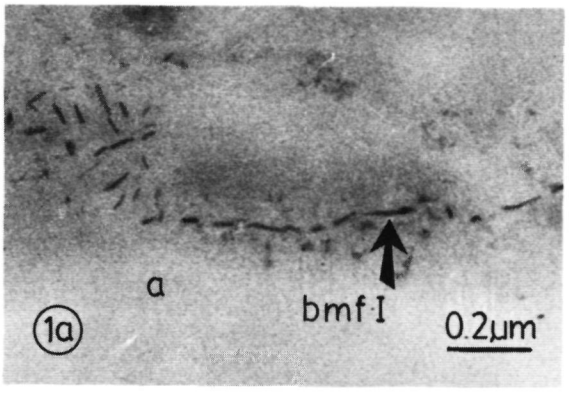
Fig. 3. Part of alveolar wall after chondroitinase ABC + *Streptomyces* hyaluronidase treatment. (a) The basement membrane filaments I are not affected by this combination of enzymes. (b) The collagen associated filaments are no longer visible.

Fig. 4. Part of alveolar wall after nitrous acid treatment. (a) The basement membrane filaments I are no longer visible. (b) The staining of the collagen associated filaments remain unaffected. abm, alveolar basement membrane; en, endothelium, cap, capillary; ep, epithelium.

Fig. 5. Collagen fibrils in the alveolar wall after pronase treatment with/without prefixation. (a) Pronase digestion after prefixation: the staining of the collagen associated filaments is not affected. (b) Pronase digestion without prefixation: the collagen associated filaments are no longer detectable.

Fig. 6. Part of the alveolar wall after treatment with 2.0 M $MgCl_2$ after prefixation. (a) The basement membrane filaments I remain visible after this treatment. (b) The collagen associated filaments also are not affected.

Fig. 7. Part of the alveolar wall after treatment with 2.0 M $MgCl_2$ without prefixation. (a) The basement membrane filaments I are not affected by this treatment. (b) The collagen associated filaments are no longer visible.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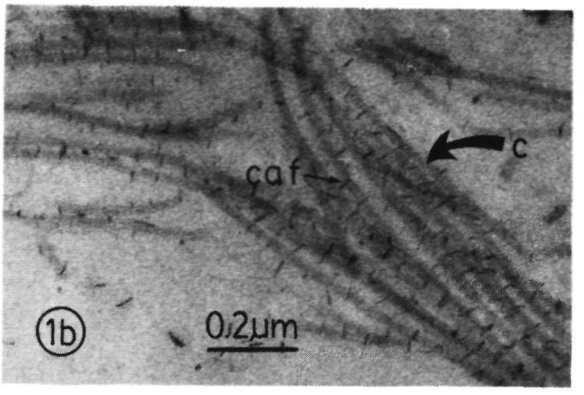
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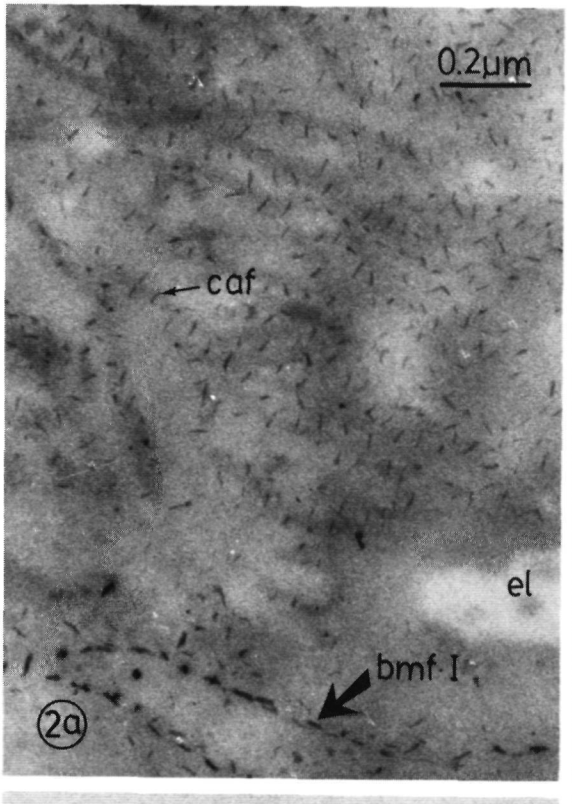
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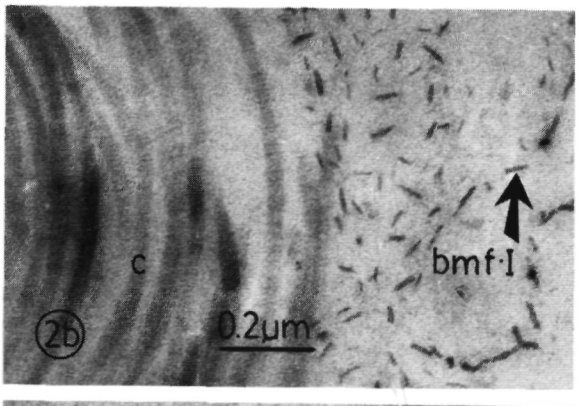
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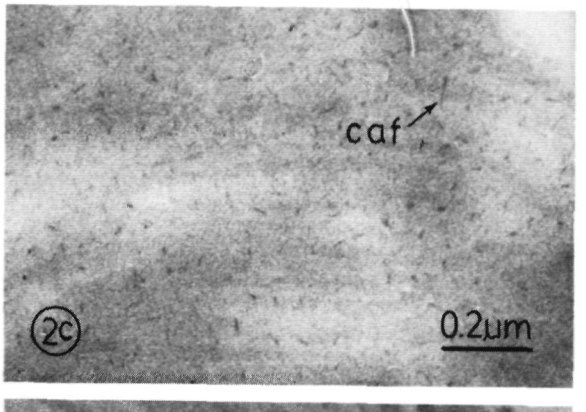
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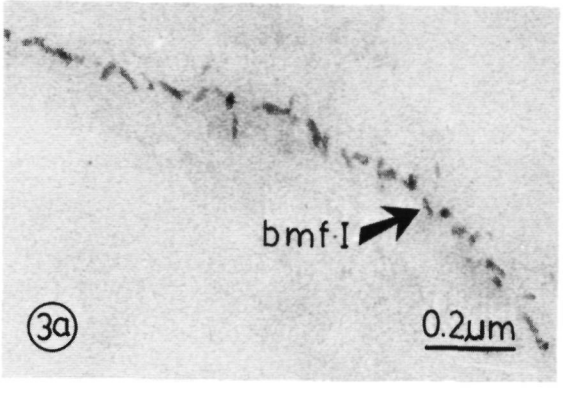
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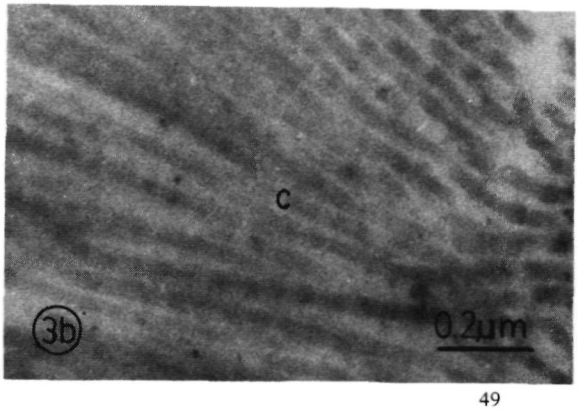
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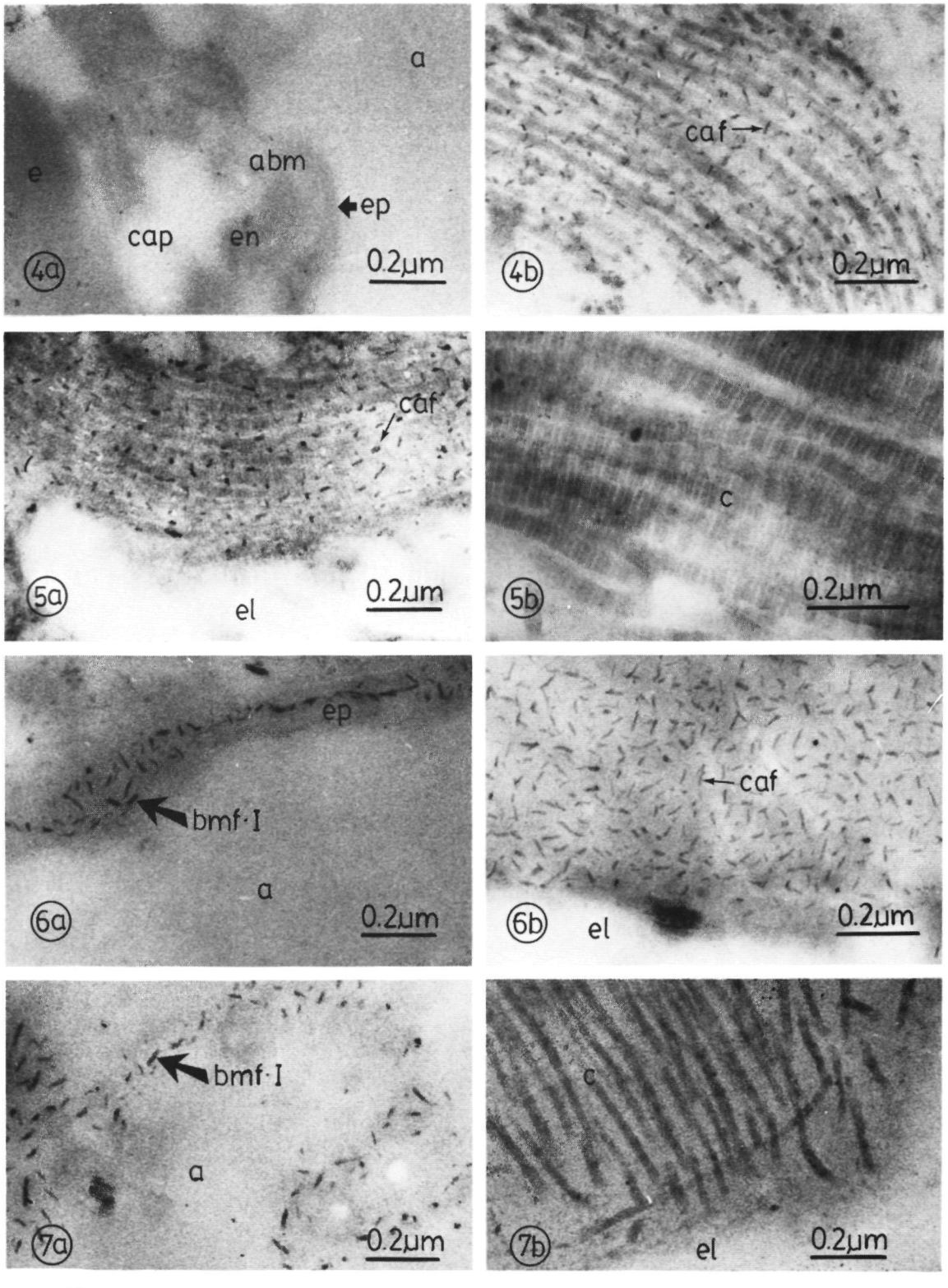
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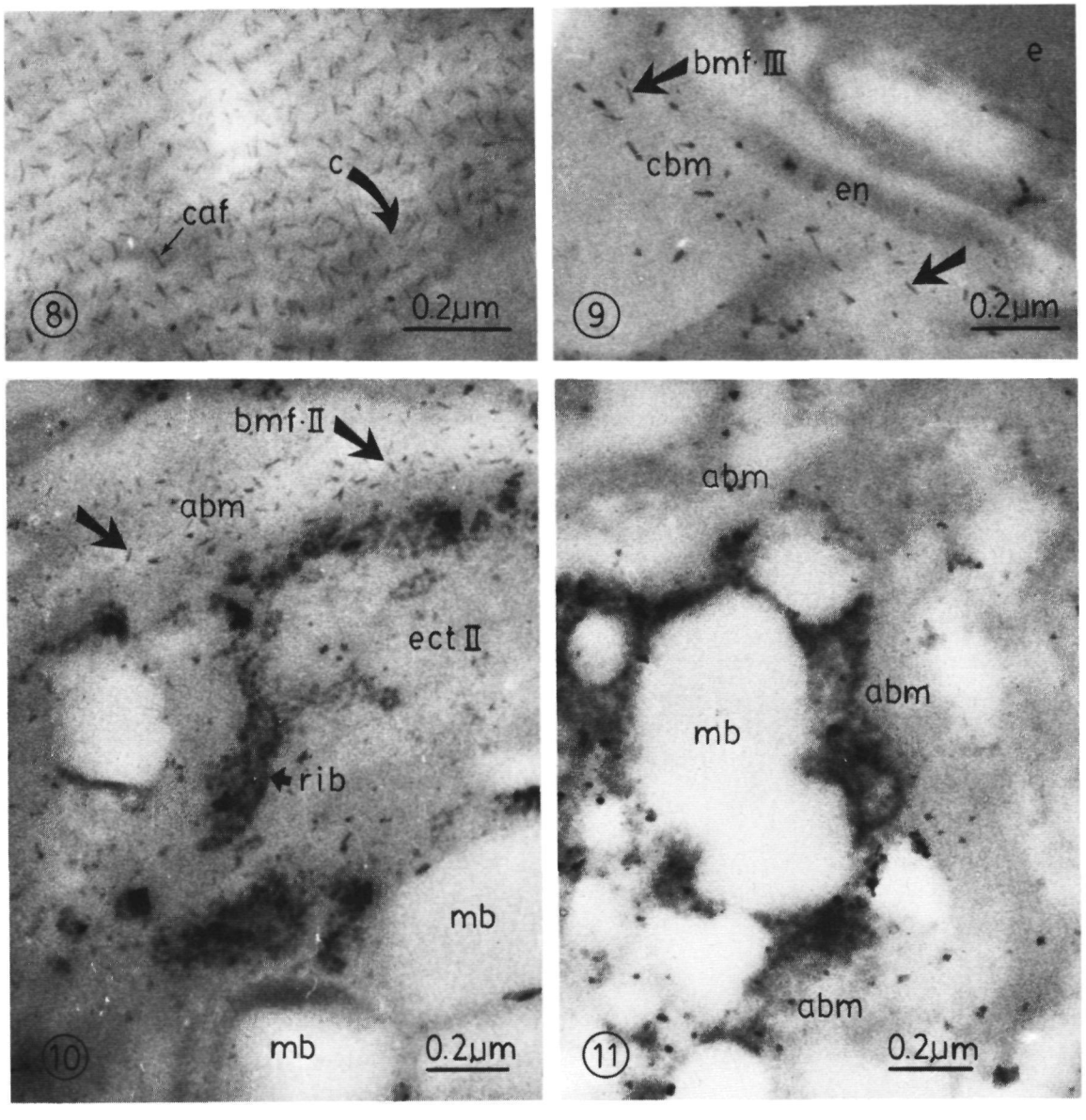

Fig. 8. Collagen fibrils in the alveolar wall after treatment with 0.2 M $MgCl_2$ without prefixation. The collagen associated filaments are not affected.

Fig. 9. Part of the alveolar wall after treatment with chondroitinase ABC. The Cuprolinic Blue-positive filaments (basement membrane filaments III) in the basement membrane of the capillary endothelium are not affected. cbm, capillary basement membrane.

Fig. 10. Part of the alveolar wall after treatment with chondroitinase ABC. The Cuprolinic Blue-positive filaments (basement membrane filaments II) in the alveolar basement membrane of type II epithelium cell (ect II) are not affected. mb, multilamellar body; rib, ribosomes.

Fig. 11. Part of alveolar wall after treatment with nitrous acid. The basement membrane filaments II cannot be detected anymore.

2.0 M is replaced by 0.2 M $MgCl_2$ the collagen associated filaments are unaffected (Fig. 8). The effects of the various treatments are summarized in Table 2.

When the stain intensity of the basement membrane filaments I is compared with that of the collagen associated filaments, the former is always the most electron dense (Fig. 2a).

The basement membrane filaments III of the endothelial cells are hardly affected by chondroitinase ABC (Fig. 9), while nitrous acid clearly affects these structures; the basement membrane filaments II of epithelial cell type II show the same sensitivity towards chondroitinase ABC (Fig. 10) and nitrous acid (Fig. 11).

Discussion

Basement membrane filaments

The Cuprolinic Blue-positive filaments in the alveolar basement membrane of type I epithelial cells disappear after nitrous acid treatment, while other glycosaminoglycan degrading procedures as well as neuraminidase digestion have no effect. This means that the stainability of the filaments is caused by either heparan sulphate or heparin or both. This is in good agreement with the results obtained for rat lung alveoli (Vaccaro & Brody, 1981). Hyaluronic acid cannot be visualized by the Cuprolinic Blue staining procedure since the 0.2 M $MgCl_2$ used during fixation prevents staining of this type of glycosaminoglycan (Scott, 1972). Hyaluronic acid, however, is probably not involved in the filaments themselves, because *Streptomyces* hyaluronidase did not have any effect on the filaments. After pronase digestion without prefixation, the filaments can no longer be detected. Application of this proteolytic enzyme, after prefixation with glutaraldehyde, has no effect. This indicates that the glycosaminoglygans are complexed with protein, but it does not elucidate the nature of the bond between the sugar and the protein. Treatment with 2.0 M $MgCl_2$ after prefixation has no effect suggesting a covalent bond between the glycosaminoglycan and the protein, since 2.0 M $MgCl_2$ will break ionic bonds. Hence, it is likely that the Cuprolinic Blue-positive filaments are proteoglycans. Each filament would represent a proteoglycan monomer since, applying a modified Cuprolinic Blue staining procedure (accompanying article), they closely resemble proteoglycan monomers with the glycosaminoglycan-chains condensed as seen using the spreading technique.

As to the involvement of heparan sulphate or heparin (or both), the following may be said. Heparan sulphate is the main glycosaminoglycan in the renal glomerular basement membrane (Kanwar & Farquhar, 1979), as well as in the basement membrane of the bovine lens capsule (Parthasarathy & Spiro, 1982). A basement membrane producing tumour also contains heparan sulphate as the predominant glycosaminoglycan (Hassell *et al.*, 1980), which is also the case for a basement membrane producing cell-line (Oohira *et al.*, 1982); glomerular epithelial cells *in vitro* have been shown to synthesize largely heparan sulphate (Farin *et al.*, 1980). Furthermore, it is known that heparin is largely confined to the mast cells (Lindahl & Höök, 1978; Nakamura *et al.*, 1981) and

recently, a good correlation has been found between the number of mast cells and the amount of heparin (Straus *et al.*, 1982). It is, therefore, likely that heparan sulphate is the only type of sulphated glycosaminoglycan in the proteoglycans of the alveolar basement membrane of type I epithelial cell. The use of the enzyme heparitinase, which degrades only heparan sulphate, would make it possible to deduce whether heparan sulphate and/or heparin are involved (Linker & Hovingh, 1972).

The heparan sulphate-containing proteoglycan visualized electron microscopically could be a similar proteoglycan to the one isolated from bovine lung gas exchange tissue by Radhakrishnamurthy *et al.* (1980); the glycosaminoglycan composition of this proteoglycan was shown to be nearly 100% heparan sulphate. Kanwar *et al.* (1981) found that only 55% of the proteoglycans synthesized was extracted from the renal glomerular basement membrane after treatment for 24 h with 4.0 M guanidinium chloride. This is in agreement with our observation that the heparan sulphate proteoglycan cannot be removed from the alveolar basement membrane by 2.0 M $MgCl_2$ without prefixation. The heparan sulphate-containing proteoglycan is apparently tightly anchored in the basement membrane.

The filaments in the alveolar basement membrane of epithelial cell type II are sensitive to nitrous acid but are chondroitinase ABC-insensitive which indicates that the stainability of these filaments is also attributable to heparan sulphate.

As described in the accompanying paper, the filaments appear in one plane in the alveolar basement membrane. This would mean that the whole alveolus contains an envelope of a single layer of heparan sulphate-containing proteoglycans, making up a space-filling structure of negative charges. This model is shown in Fig. 12. A filtering function seems likely for this structure, a function which has also been recognized for the heparan sulphate-containing proteoglycans of the renal glomerular basement

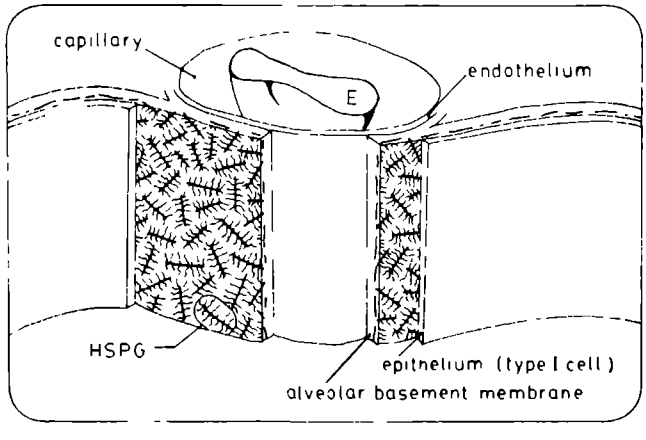

Fig. 12. Schematic representation of the heparan sulphate containing proteoglycan-envelope in the alveolar basement membrane. E, erythrocyte; HSPG: heparan sulphate containing proteoglycan.

membrane (Kanwar *et al* , 1981), removal of the glycosaminoglycans leads to increased permeability of the glomerular basement membrane to serum albumin (Kanwar & Farquhar, 1980) This filter could play a role in regulating the transport of biomolecules from the blood to the epithelium (and eventually to the alveolar airspace) and vice versa Transport of fluid from the airspace into the interstitium has been suggested (Matthay *et al* , 1982, Olver *et al* , 1981)

Another role of this negative sheet of heparan sulphate-containing proteoglycans might be in reducing the effect of the surface tension at the alveolar–air interface It is well established that the surfactant system contains surface-active phospholipids, which reduce the surface tension of the alveoli, thereby preventing a collapse Due to the mutual repulsion of their negative charges, the heparan sulphate-containing proteoglycan shell may also have a function in defining the minimal diameter of the alveolus, thereby playing an important role in the physiological functioning of the lung

The basement membrane of the capillary endothelial cells contains Cuprolinic Blue-positive filaments which are also nitrous acid-sensitive, but are hardly affected by chrondroitinase ABC digestion, heparan sulphate therefore is the predominant glycosaminoglycan This agrees with the studies of Clowes *et al* (1982) who, using antibodies to a heparan sulphate-containing proteoglycan, showed the rat capillary endothelial basement membrane to be a major site of such proteoglycans Furthermore, Cantor *et al* (1977) demonstrated that rat lung endothelial cells *in vitro* may secrete heparan sulphate Vaccaro & Brody (1981) showed that Ruthenium Red-positive granules in the basement membrane of rat lung alveoli contained heparan sulphate

Collagen associated filaments

The collagen fibril associated filaments disappear completely after chondroitinase ABC digestion, but they remain after nitrous acid treatment One can conclude from these observations that the stainability of the filaments arises from the presence of either dermatan sulphate or chondroitin sulphate or both as has also been observed earlier for rat lung (Vaccaro & Brody, 1979) and kidney (Farquhar & Kanwar, 1982) The use of the enzyme chondroitinase AC results in a decrease, but not in a disappearance, of the electron density of the filaments The remaining electron density has to be attributable to dermatan sulphate, the electron density lost, however, may be either chondroitin sulphate or glucuronic acid residues containing dermatan sulphate or both After pronase digestion without prefixation, the filaments are no longer detectable, pronase digestion after fixation, however, has no effect Treatment with 2 0 M $MgCl_2$ after prefixation, also has no effect Therefore the collagen associated filaments represent proteoglycan monomers with dermatan sulphate (and possibly chondroitin sulphate) as the glycosaminoglycan component This dermatan sulphate-containing proteoglycan is less electron dense than the heparan sulphate-containing proteoglycan of the alveolar basement membrane This could be due to more glycosaminoglycan chains per length of the heparan sulphate proteoglycan-filament, more sulphate groups per disaccharide group of the heparan sulphate chains and/or longer heparan sulphate chains

Scott and his colleagues (1981) demonstrated in a comparative electron microscopical and biochemical study that small foetal collagen fibrils in rat tail tendon seemed to be preferentially associated with chondroitin sulphate and hyaluronic acid, while for coarse collagen fibrils dermatan sulphate was the predominant glycosaminoglycan. Our observation, that some areas are less chondroitinase AC-sensitive might, therefore, reflect areas where the proteoglycans contain more dermatan sulphate. A correlation of different glycosaminoglycans with specific types of collagen has been suggested (Junqueira *et al.*, 1981).

Fibroblasts might be the site of the dermatan sulphate-containing proteoglycan production since it has been shown (Castor *et al.*, 1979) that the major sulphated glycosaminoglycan synthesized by guinea-pig lung fibroblasts in culture was dermatan sulphate. Furthermore, Ehrlich (1981) demonstrated that fibroblast-like lung cells of the rat in culture secreted a dermatan sulphate/chondroitin sulphate-containing proteoglycan in the medium, while human embryonic lung fibroblasts in culture secreted dermatan sulphate proteoglycans as the major proteoglycan fraction (Vogel & Peterson, 1981).

The nature of the interaction of the dermatan sulphate proteoglycan with the collagen fibrils is probably ionic since after treatment with 2 0 M $MgCl_2$ without prefixation this proteoglycan can no longer be observed. This is in agreement with the studies of several authors who demonstrated that the interaction of glycosaminoglycans and proteoglycans with collagen can be abolished by increasing the ionic strength (Mathews, 1965; Obrink, 1973a; Greenwald *et al.*, 1975).

As described earlier, the correspondence of the distance between the filaments and the collagen fibril main banding pattern is an indication of a specific structural interaction between these two components of the connective tissue. It has been demonstrated by Obrink (1973a) that dermatan sulphate and dermatan sulphate-containing proteoglycan can interact with collagen; dermatan sulphate binds to (lathyritic) collagen more strongly than chondroitin sulphate, the iduronic acid residue being an important determinant in the strength of binding. A strong interaction of dermatan sulphate with collagen has been noted previously by Toole & Lowther (1968); dermatan sulphate extraction from tendon and heart valves was far more difficult than extraction of other glycosaminoglycans. Furthermore, Wusteman (1972) has demonstrated for bovine lung parenchyma, that dermatan sulphate is closely associated with collagen fibrils, since collagenase digestion removed more dermatan sulphate than either salt extraction or elastase digestion. Possibly the dermatan sulphate-containing proteoglycan maintains the interconnection of the collagen fibrils in lung alveoli and in such a way provides structural coherence; the localization of this proteoglycan, as visualized in this study, is consistent with this view.

Another role of this proteoglycan might be in fibrillogenesis. Dermatan sulphate, as well as dermatan sulphate-containing proteoglycan, can precipitate collagen molecules (Obrink, 1973a), while both have an effect on fibril formation (Obrink, 1973b), Snowden & Swann (1980) also noticed a marked effect of dermatan sulphate on fibril formation, in

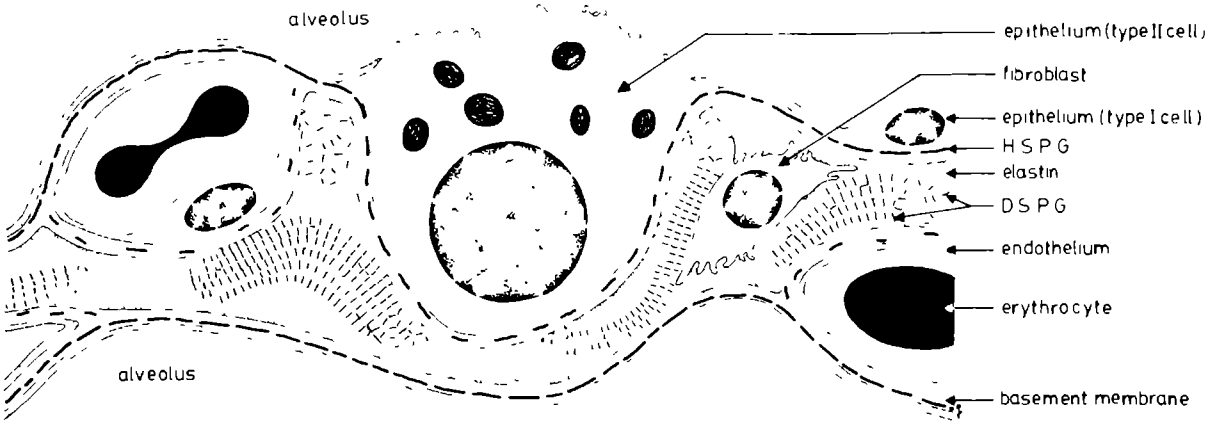

Fig. 13. Schematic representation of the localization of the various proteoglycans in mouse lung alveoli seen after applying the Cuprolinic Blue staining procedure. HSPG, heparan sulphate containing proteoglycan; DSPG, dermatan sulphate containing proteoglycan.

contrast with chondroitin-6-sulphate, which had a much smaller influence. Furthermore, dermatan sulphate seems to be associated with the more coarse collagen fibrils (Scott *et al.*, 1981; Eyre & Muir, 1975); an increase in dermatan sulphate content, prior to an increase of collagen deposition has also been noticed (Kawamoto & Nagai, 1976), as well as an influence of dermatan sulphate on the mechanical properties of collagen fibrils (Danielson, 1982). Hence, it may be possible that the dermatan sulphate-containing proteoglycan plays a role in the fibrillogenesis of collagen fibrils in lung alveoli. Moreover, a shift in glycosaminoglycan composition during lung development (Horwitz & Crystal, 1975) may reflect alterations in collagen fibrils, as already shown for tendon (Scott *et al.*, 1981). It seems plausible that this proteoglycan plays a role not only in the development of collagen fibrils in lung, but also in determining the tensile strength of these fibrils once formed. A disturbance in the amount or nature of these proteoglycans could well lead to a loss of stability of the alveoli, thus leading to pathological conditions such as emphysema.

Fig. 13 gives a schematic representation of the localization of the various proteoglycans in mouse lung alveoli.

Acknowledgement

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CHAPTER 4

Localization of proteoglycans in tissue using the critical electrolyte concentration method. Comparison with biochemical data from literature.

TOIN H.M.S.M. VAN KUPPEVELT, TWAN L.M. RUTTEN AND CHARLES M.A. KUYPER

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Summary

Several connective tissues were stained for proteoglycans using the cationic dye Cuprolinic Blue according to the critical electrolyte concentration method. This method visualizes proteoglycans as electron-dense filaments. In most tissues two types of proteoglycan-filaments are present: a small (maximum length 60 nm), thin, collagen fibril-associated filament, and a thick, heavily staining filament which is predominately localized between bundles of collagen fibrils. Cartilage contains very large (about 300 nm) proteoglycan-filaments while in cornea they are very small. Comparison with biochemical data from literature suggests that the appearance of the proteoglycan-filaments may be indicative for the glycosaminoglycan/protein ratio and for the molecular weight of the part of the protein core to which glycosaminoglycans are attached. The data thus obtained on the localization and structure of a proteoglycan may be useful when planning a strategy for its isolation.

Introduction

Connective tissue is characterized by a relatively high amount of extracellular matrix. In this matrix a fibrillar and an amorfous component can be distinguished; collagen and elastin are elements of the former, while proteoglycans are major elements of the latter. Proteoglycans are macromolecules consisting of a protein core to which glycosaminoglycans are covalently attached in addition to (in most cases) some oligosaccharides (Hascall & Hascall, 1981). By virtue of carboxylic and sulfate groups the glycosaminoglycans (and hence the proteoglycans) are highly negatively charged molecules. There is a great variety of proteoglycans and several physiological functions such as acting as a filter barrier (Kanwar et al., 1980) and absorbing compressive load (Hascall & Hascall, 1981) have been ascribed to them.

There are several ways for the ultrastructural detection of proteoglycans in tissue. The most commonly used method is the application of cationic probes such as Alcian Blue (Goldberg et al., 1978), Ruthenium Red (Gordon & Bernfield, 1980) and cationized ferritin (Kanwar & Farquhar, 1979). The interaction between the dye and the proteoglycan is probably of an ionic nature (Scott, 1973) and consequently the specificity of these stains is not very high (i.e. all negatively charged molecules may bind the dye). Specificity, however, can be increased when the critical electrolyte concentration method is used (Scott, 1980). In this method the staining solution contains a salt (e.g. $MgCl_2$) in such a molarity that only sulfate groups can bind the dye and hence only sulfated molecules are stained. In practice this means that in the extracellular matrix proteoglycans are about the only components to be contrasted.

Another method for the ultrastructural detection of proteoglycans is the application of antibodies raised against a particular proteoglycan (Longas and Fleischmajer, 1985; Aquino et al., 1984). Although the specificity of this method is better than that using cationic probes, resolution is not. Phtalocyanin-like dyes (e.g. Alcian Blue, Curpolinic Blue, Cupromeronic Blue) used according to the critical electrolyte concnetration method allow an accurate localization of proteoglycans in tissue (Ruggeri et al., 1975; Scott, 1981; Van Kuppevelt et al., 1984a,b, 1985a). Furthermore, addition of cationic dyes to the fixative reduces the loss of proteoglycans during tissue processing to less than 1% (Chen & Wight, 1984).

In this study proteoglycans are localized in several connective tissues using Cuprolinic Blue according to the critical electrolyte concentration method; an attempt will be made to correlate ultrastructural observations with biochemical data from the literature.

Materials

Bovine flexor tendon, achilles tendon, nasal septum cartilage, ear cartilage, cornea, skin and periodontal ligament were obtained from the local slaughterhouse. Cornea from New Zealand White rabbits and skin from female Sprague-Dawley rats were obtained from the Central Animal Laboratory, University of Nijmegen. Samples were taken at random. After dissection, the specimens were immediately put into the fixative. Reconstituted collagen fibrils with and without small dermatan sulfate proteoglycans (Danielsen and Uldbjerg, 1983; Danielsen, 1982) were a kind gift of Dr. C.C. Danielsen, University of Aarhus, Denmark.

Methods

The Cuprolinic Blue staining procedure has been outlined previously (Van Kuppevelt et al., 1985a). Briefly, specimens were fixed overnight (room temperature) in 0.05 M sodium acetate (pH 5.6) containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue and 0.2 M $MgCl_2$. After rinsing, the tissue blocks were further stained with 1% aqueous Na_2WO_4 and dehydrated in ascending ethanol concentrations, the 30 and 50% containing 1% Na_2WO_4. Embedding was in Epon 812. Sections (± 80 nm) were cut with a diamond knive and poststained with 2% aqueous uranyl acetate. Sections were examined with a Philips 201 electron microscope; the magnification was calibrated using a cross grating replica (2160 lines/mm; Balzers Union, Liechtenstein).

Results

It has been well documented (Scott and Orford, 1981; Iozzo, 1984; Van Kuppevelt et al., 1984a,b, 1985a), and some additional proof will be presented below, that the electron-dense filaments in tissue appearing after the Cuprolinic Blue staining procedure, represent proteoglycans (PG); the Cuprolinic Blue-positive filaments will therefore be referred to as PG-filaments.

There are two main problems in estimating the real length of PG-filaments.
1. A filament may lie obliquely with respect to the plane of sectioning; the measured length then is smaller than the real length. The use of a goniometer device did not satisfactorily solve this problem.
2. A filament may be only partially included in the section; in this case also the measured length is less than the actual length of the filament.
In this study the maximum length observed within a class of filaments was taken as the real length. In such way, however, a genuine class of small PG-filaments may be overlooked. Only regularly occuring PG-filaments associated with or in the vicinity of collagen fibrils were studied here. Other PG-filaments, likely to occur in distinct areas of the tissue, were not taken into consideration.

Reconstituted collagen fibrils with dermatan sulfate proteoglycans

Proteoglycans appear as electron-dense filaments after the Cuprolinic Blue staining procedure (Fig. 1). Their maximum length is about 60 nm; large aggregates may be present. In the control (i.e. no proteoglycans added) no such structures can be observed (Fig. 2).

Tendon

The PG-filaments of tendon (Fig. 3) can be distinguished into at least two major classes; one class consists of thin, small (maximum length about 60-70

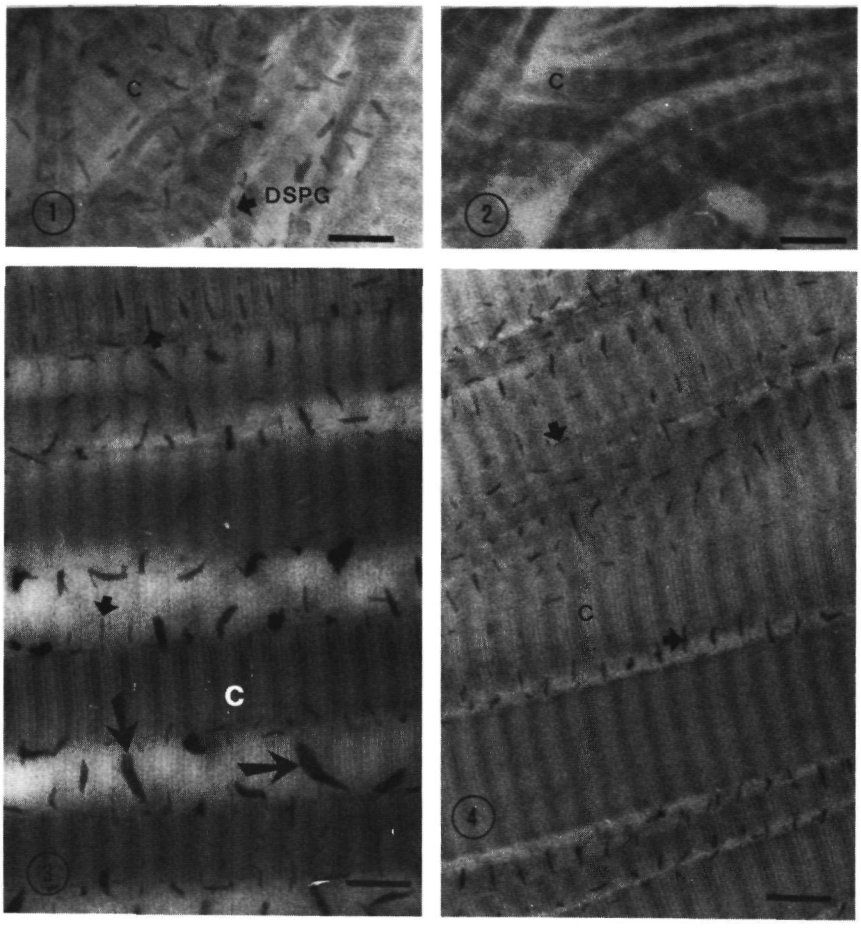

Fig. 1. Reconstituted collagen fibrils (c) to which dermatan sulfate proteoglycans (DSPG) have been added. Cuprolinic Blue staining procedure. The proteoglycans appear as electron-dense filaments. Bar: 150 nm.

Fig. 2. Reconstituted collagen fibrils (c). No proteoglycans added. Cuprolinic Blue staining procedure. No electron-dense filaments are present (compare with Fig. 1). Bar: 150 nm.

Fig. 3. Bovine achilles tendon after the Cuprolinic Blue staining procedure. Thin, small proteoglycan (PG)-filaments (small arrows) are present in addition to larger, thick, heavily staining PG-filaments (large arrows). The small PG-filaments can be seen regularly associated with collagen fibrils (c). Bar: 150 nm.

Fig. 4. Bovine sclera after the Cuprolinic Blue staining procedure. Thin, small PG-filaments (arrows) are visible. The ones orientated perpendicularly with respect to the collagen fibril (c) axis are predominately associated with the d-band of the fibril. Bar: 150 nm.

nm) PG-filaments while the other class consists of larger, thick and heavily staining PG-filaments which are preferentially localized around bundles of collagen fibrils or at places where the collagen fibrils are somewhat separated from each other. PG-filaments running parallel as well as perpendicular with respect to the collagen fibrils are present. Sometimes extraordinarily large PG-filaments (upto 200 nm) can be observed; they lie in a parallel fashion with respect to the collagen fibrils.

Sclera

The coarse collagen fibrils of the sclera are also associated with small, thin PG-filaments with a maximum length of 50-60 nm (Fig. 4), similar to those found in human and rabbit sclera (Young, 1985). Between bundles of collagen fibrils thick, heavily staining filaments are present.

Periodontal ligament

In this tissue also, two major classes can be distinguished; a class of small, thin, collagen fibril-associated, PG-filaments (maximum length about 60 nm) and a class of larger, thick and heavily staining PG-filaments, which are localized between bundles of collagen fibrils (Fig. 5).

Skin

The collagen fibrils of skin are associated with small, thin PG-filaments (Fig. 6) with a maximum length of 50-60 nm. Furthermore, capricious PG-filaments can be sometimes detected, which are mostly localized at places where bundles of collagen fibrils of different orientation meet (Fig. 5). Especially in the skin of the rat a very small PG-filament seems to be present.

Cornea

Sections of cornea present a somewhat different image when compared to other

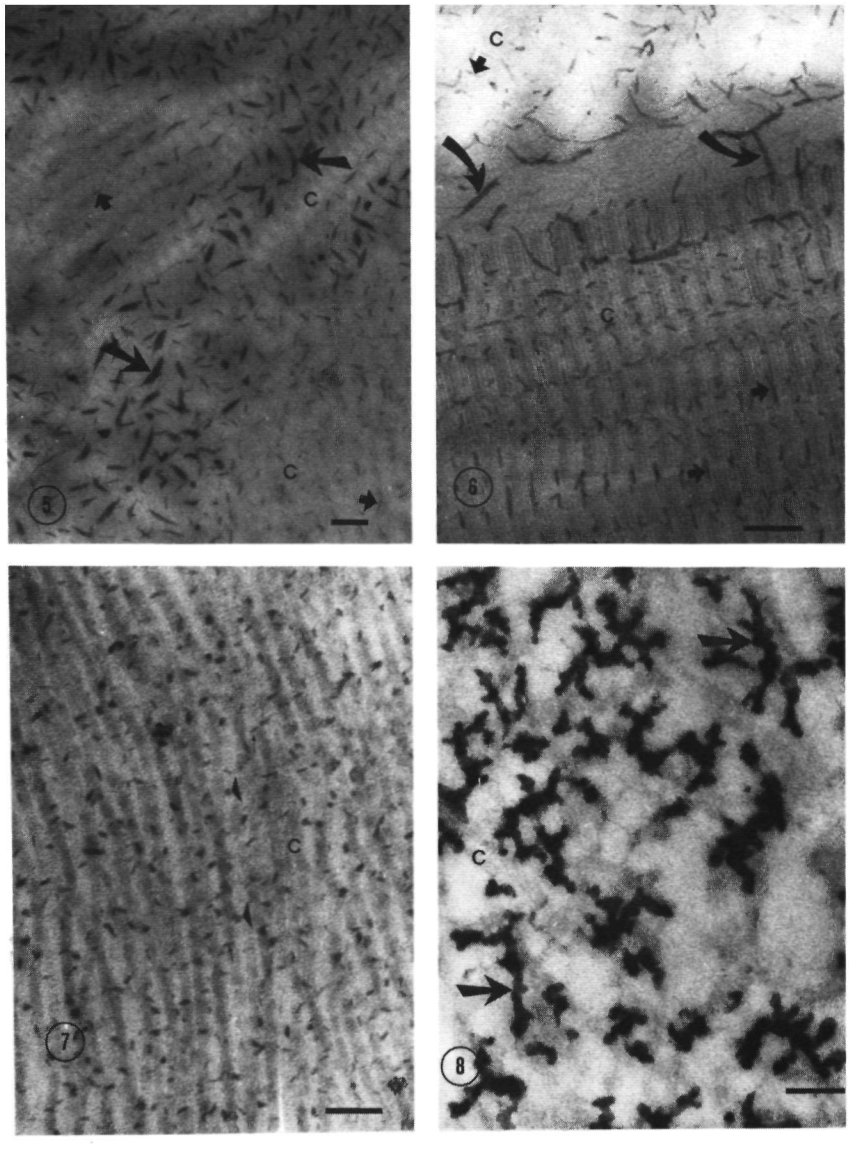

Fig. 5 Bovine periodontal ligament after the Cuprolinic Blue staining procedure. Thin, small PG-filaments (small arrows) are associated with collagen fibrils (c). Larger, thick, heavily staining PG-filaments (large arrows) have an inter-fibrillar localization. Bar: 150 nm.

Fig. 6. Rat skin after the Cuprolinic Blue staining procedure. Small, thin PG-filaments (smaal arrows) are detectable. At places where bundles of collagen fibrils (c) of different orientation meet, capricious PG-filaments (large arrows) are present. Bar: 150 nm.

Fig. 7. Bovine cornea after the Cuprolinic Blue staining procedure. Dot-like PG-"filaments" (triangles) are visible in addition to very small PG-filaments (small arrows). c: collagen fibril. Bar: 150 nm.

Fig. 8. Bovine ear cartilage after the Cuprolinic Blue Staining procedure. Very large, thick, heavily staining PG-filaments (large arrows) are visible. c: collagen fibril. Bar: 150 nm.

tissues. The PG-filaments are very small and often appear as dot like structures (Fig. 7). A major class of about 30 nm is present. A considerable part, however, seems to be in a genuine globular state. Another class, which is present in a low proportion, has a maximum length of about 50 nm.

Cartilage

The PG-filaments of cartilage (Fig. 8) appear as irregular structures which are very large (maximum length about 300 nm), thick and intensely stained. No direct relation to the collagen fibrils seems to be present. Occasionally large aggregates of these PG-filaments can be observed. Sometimes small PG-filaments are seen associated with collagen fibrils.

It was determined that for tendon, sclera and skin the small, thin PG-filaments which are orientated perpendicular with respect to the collagen fibrils, are predominately (70-80%) located at the d-band of the collagen fibril.

The Cuprolinic Blue staining procedure reveals proteoglycan monomers as electron-dense filamentous structures, formed by the collapse of the glycosaminoglycan side-chains onto the protein core during specimen processing (Van Kuppevelt et al., 1984a,b, 1985a; Scott, 1980, Figs. 1 and 2). These structures, being reflections of intact proteoglycans, may therefore contain some information regarding the chemical nature of the proteoglycans.

A small, thin PG-filament with a maximum length of about 60 nm is abundantly present in most connective tissues studied here; those orientated perpendicularly with respect to the collagen fibril axis are predominately associated with the d-band of the collagen fibrils. The dermatan sulfate proteoglycans added to the reconstituted collagen fibrils (Fig. 1) also belongs to this class, although no specific d-band association could be observed. This proteoglycan, isolated from human uterine cervix (Uldbjerg et al., 1983) belongs to a group of proteoglycans named PG-Sm-DS according to the nomenclature suggested by Heinegård and co-workers (1985) in which PG stands for proteoglycan, Sm for small and DS for dermatan sulfate. This class of proteoglycans is characterized by a M_r of around or less than $100 \cdot 10^3$, a protein core of $40\text{-}60 \cdot 10^3$ with high contents of leucine, aspartic acid/asparagine and glutamic acid/glutamine, the core bearing one or only few dermatan sulfate side-chains. Small proteoglycans isolated from bovine achillus tendon (Anderson, 1975) and flexor tendon (Vogel and Heinegård, 1985), sclera (Cöster and Fransson, 1981), skin and periodontal ligament (Pearson and Gibson, 1982) with such characteristics have been described. The dermatan sulfate proteoglycan from rat skin (Miyamoto and Nagese, 1980), however, seems to have a very small M_r. We suggest that the small, thin PG-filaments visualized by the Cuprolinic Blue staining procedure, are the electron microscopical reflections of the PG-Sm class. These proteoglycans are associated with coarse collagen fibrils (tendon, sclera)

as well as with smaller collagen fibrils (skin, periodontal ligament). A role in regulating the diameter of a collagen fibril seems therefore questionable; they can, however, be of importance in inhibiting the lateral growth of a fibril. The observation of Vogel and Heinegård (1983) that addition of a small dermatan sulfate proteoglycan to a collagen solution leads to a reduced increase of absorbance, may be explained in this way.

The corneal stroma contains smaller PG-filaments (Fig. 7) and a part of the proteoglycans appear as globular structures. It has been reported for rabbit cornea (Gregory et al., 1982) that four different proteoglycans are present: two keratan sufate proteoglycans and two dermatan sulfate proteoglycans, the latter being larger than the former. A keratan sulfate proteoglycan from bovine corneal stroma has been shown to contain intrachain disulfide bonds (Axelsson and Heinegård, 1978); cleavage of the disulfide bonds altered the size of the molecule. The dot-like appearance of a part of the proteoglycans in our study may therefore be caused by the folding of this proteoglycan into a globular structure.

For bovine tendon (Vogel et al., 1985; Anderson, 1975), sclera (Cöster and Fransson, 1981) and periodontal ligament (Pearson and Gibson, 1982) it has been described that besides a small proteoglycan, a larger proteoglycan is present with a higher glycosaminoglycan/protein ratio. For periodontal ligament it was suggested that this proteoglycan has an interfibrillar localization, a place where we find thick heavily staining PG-filaments. We suggest that the thick, heavily staining PG-filaments represent this class of proteoglycans, their electron microscopical appearance being the result of the higher glycosaminoglycan/protein ratio, i.e. more glycosaminoglycan side-chains attached to the protein core. The main proteoglycan from cartilage which possesses about 130 glycosaminoglycan side-chains also appears as a thick heavily staining filament (Fig. 8). Its irregular shape is probably the result of an incomplete collapse of the glycosaminoglycans onto the core. Interestingly, Iozzo (1984)

isolated a heparan sulfate proteoglycan (M_r about $950 \cdot 10^3$; M_r core protein about $240 \cdot 10^3$), which appears as a long (maximum length 220 nm), thin filament after the Cuprolinic Blue staining procedure. The stain intensity of the filament is considerably less when compared to that of the cartilage proteoglycan, which has a core protein of similar M_r (about $200 \cdot 10^3$) but a larger M_r ($2000\text{-}3000 \cdot 10^3$) and a higher glycosaminoglycan/protein ratio (Hascall and Kimura, 1982; Schwarz et al., 1985) explaining the difference in staining capacity. The ultrastructural appearance of a PG-filament may therefore give an indication of the glycosaminoglycan/protein ratio of a proteoglycan.

The length of a PG-filament may give an indication of the molecular weight of the part of the protein core to which the glycosaminoglycans are attached. It should be noted that in the case of small proteoglycans the glycosaminoglycan side-chain(s) will occupy a large part of the protein core after collapse, even when only one glycosaminoglycan side-chain is present at the end of the protein core as is the case in most dermatan sulfate proteoglycans from bovine skin (Chopra et al., 1985). Taking a molecular weight of 110Da and a length of 0.2 nm per amino acid residue (Hascall, 1980), the protein core of the small proteoglycan (length about 60 nm) would have a M_r of $33 \cdot 10^3$. This value approaches the M_r obtained for the whole protein core by biochemical methods (sodium dodecyl sulfate polyacrylamide-gel electroforesis), which is $40\text{-}50 \cdot 10^3$. The cartilage proteoglycan has a maximum length of about 300 nm which is similar to the value obtained for spreaded molecules (Thyberg et al., 1975). The molecular weight of the glycosaminoglycan-decorated part of the protein core would therefore be $165 \cdot 10^3$. It can be calculated that the M_r obtained by biochemical methods is about $150 \cdot 10^3$ (Hascall and Kimura, 1982). The glycosaminoglycan associated part of the protein core of a proteoglycan from human colon carcinoma cells (maximum length 220 nm, Iozzo, 1984) would have a molecular weight of $121 \cdot 10^3$. The M_r of the whole protein core is however about $240 \cdot 10^3$, suggesting that only a part of the core is occupied by

the glycosaminoglycans, as in cartilage. This may be due to its localization at the cell surface; a part of the core protein is possibly inserted into the cell membrane. However, one has to be careful in applying such calculations. For instance, the protein core may be folded (see above); furthermore it has been shown for a basement membrane proteoglycan from mouse alveoli (but not from human alveoli, Van Kuppevelt et al., 1984a, 1985a) that the length of the PG-filament is dependent on the $MgCl_2$ concentration used during staining.

The Cuprolinic Blue staining procedure might also give a clue as to which extraction solution may be used when a specific proteoglycan has to be isolated. Interfibrillar proteoglycans can be extracted by use of 0.1 M NaCl (Pearson and Gibson, 1982), while collagen fibril-associated dermatan sulfate proteoglycans require harder methods such as treatment with 4 M guanidine HCl (Damle et al., 1982; Vogel and Heinegård, 1985) or 2 M $CaCl_2$ (Van Kuppevelt et al., 1985b). Cell associated proteoglycans can be solubilized by the use of a detergent or by mild trypsinization (Iozzo, 1984).

In conclusion the Cuprolinic Blue staining procedure may be useful not only in precisely localizing proteoglycans in tissue but also in indicating the apparant molecular weight of the part of the protein core to which glycosaminoglycans are attached and the glycosaminoglycan/protein ratio. Such data may be useful in planning a strategy for the isolation of a specific proteoglycan.

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CHAPTER 5

Ultrastructural localization and characterization of proteoglycans in human lung alveoli.

TOIN H.M.S.M. VAN KUPPEVELT, FRANS P.M. CREMERS, JOS G.W. DOMEN, HENK M. VAN BEUNINGEN, ADRIAAN J.C. VAN DEN BRULE AND CHARLES M.A. KUYPER

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Ultrastructural localization and characterization of proteoglycans in human lung alveoli

Tom H M S M van Kuppevelt[1]), Frans P M Cremers, Jos G W Domen, Henk M van Beuningen, Adriaan J C van den Brule, Charles M A Kuyper

Department of Chemical Cytology, Faculty of Science, University of Nijmegen The Netherlands

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Lung alveoli — basement membrane — collagen — proteoglycans

In order to localize and characterize proteoglycans in human lung alveoli, we have used the cationic dye Cuprolinic Blue according to the critical electrolyte concentration method After staining, five types of Cuprolinic Blue positive filaments become apparent two types in the basement membranes of type I and type II epithelial cells respectively and lying in one or two layers one type, more scattered, localized in the basement membrane of the endothelial cells and another kind associated with collagen fibrils and separated from each other according to the main banding period (±60 nm) of these fibrils Finally, there was a type of filament which was only locally present at a variety of places The basement membrane filaments were sensitive to heparinase, heparitinase, pronase (without prefixation) and nitrous acid treatment, but not to Streptomyces hyaluronidase, neuraminidase, chondroitinase ABC, chondroitinase AC, pronase (after prefixation) and 2 0 M $MgCl_2$ treatment The basement membrane filaments, therefore, represent heparan sulphate containing proteoglycans On the other hand, the collagen fibril associated filaments were sensitive to treatment with heparinase, chondroitinase ABC and pronase (without prefixation), but insensitive to Streptomyces hyaluronidase, neuraminidase, nitrous acid, heparitinase, chondroitinase AC, pronase (after prefixation) and 2 0 M $MgCl_2$ (after prefixation) treatment These filaments thus represent iduronic acid-rich dermatan sulphate containing proteoglycans Several physiological functions for these proteoglycans are discussed

Introduction

There are seven types of glycosaminoglycans (GAGs) hyaluronic acid, dermatan sulphate, chondroitin-4-sulphate, chondroitin-6-sulphate, heparan sulphate, heparin and keratan sulphate GAGs are polysaccharides consisting of repeating disaccharides of which one is an amino sugar, the other an uronic acid (with the exeption of keratan sulphate, which lacks uronic acid) All seven types of GAGs are present in the lung [8, 27], keratan sulphate being restricted to only the tracheobronchial cartilage [9] In tissue the GAGs (except probably hyaluronic acid) are covalently bound to a protein core, thus forming the so called proteoglycans (PGs) Among the functions ascribed to PGs are those of acting as a filter barrier [11], playing a role in morphogenesis [17] and absorbing compressive load [7] Histochemical studies at the electron microscopic level make the precise localization of PGs in tissue possible, thereby providing information which may greatly attribute to the elucidation of the function of the PG under study

PGs (GAGs) are thought to play a role in several pathological conditions For lung, changes in GAGs have been shown to occur in pulmonary emphysema [13, 16] and pulmonary fibrosis [1, 24], data were obtained from biochemical studies However, such studies are hampered due to the structural complexity of the lung Alveoli, small blood vessels and bronchioli are completely integrated, making it impossible to estimate the GAG-content of the separate elements properly This may explain the conflicting data concerning changes in GAG-content in pulmonary emphysema Histochemical studies do not have these limitations and may therefore be useful when studying pathological conditions

To localize PGs in human lung alveoli, we have used the cationic phthalocyanin-like dye Cuprolinic Blue according to the critical electrolyte concentration method [28] Previous studies [33, 34] have shown that this method is very well suited for localizing PGs at the electron microscopic level

Materials and methods

Small lung blocks (± 1 mm^3) were obtained from lung lobes immediately after lobectomy or pneumonectomy The samples were taken from peripheral lung tissue, not showing any sign of the process for which the patient was operated (mostly a central carcinoma) The lung blocks were degassed in the first solution which they were placed in, in order to obtain good penetration of the solution

Enzyme nitrous acid and $MgCl_2$ treatment

Unless stated otherwise, treatment with enzymes and nitrous acid is preceded by prefixation of the lung blocks for 2 h (4 °C) in 0 025 M sodium acetate buffer (*p*H 5 6), containing 2 5% glutaraldehyde Pilot experiments have shown that prefixation does not alter the effects of the glycosidases or nitrous acid, the lung ultra-

[1]) Drs Tom H M S M van Kuppevelt, Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen/The Netherlands

structure, however, is better preserved if prefixation is applied. The procedures for treatment with Streptomyces hyaluronidase, neuraminidase, chondroitinase ABC, chondroitinase AC, pronase, nitrous acid or $MgCl_2$ have been outlined previously [34]; chondroitinase AC was also used at a concentration of 2 U/ml. In the enzyme controls the enzyme has been omitted; as a control for nitrous acid treatment a 0.2 M sodium acetate solution with the same *p*H as the nitrous acid solution was taken.

Digestion with crude heparinase (a kind gift from Dr. A. Linker, University of Utah) was performed in 0.1 N sodium acetate buffer (*p*H 7.0) containing 2 mg heparinase/ml for 90 min at 37 °C. In order to prevent damage to the tissue due to proteolytic enzymes either from the tissue itself or as contaminants from the enzyme applied, 5 mM benzamidine HCl (Aldrich-Europe, Beerse, Belgium) and 0.1 M 6-amino-n-caproic acid (Janssen Chimica, Beerse, Belgium) were added. After incubation, the specimen was washed once (10 min, 0 °C) in the solution without enzyme and once (10 min, 0 °C) in 0.025 M sodium acetate buffer (*p*H 5.6) containing 0.2 M $MgCl_2$ and protease inhibitors.

Digestion with heparitinase (Seikagaku Kogyo Co., Tokyo, Japan) was as follows. After prefixation the lung blocks were washed twice (10 min, 0 °C) in 0.1 N sodium acetate (*p*H 7.0) containing 10 mM CaAc and protease inhibitors. Incubation (2 h, 43 °C) was performed in the same solution containing 10 U heparitinase/ml. Next, the tissue was washed once (10 min, 0 °C) in the solution without enzyme and once in 0.025 M sodium acetate buffer (*p*H 5.6) containing 0.2 M $MgCl_2$ and protease inhibitors.

The substrate(s) of all the treatments employed are given in Table I. After the enzyme, nitrous acid or $MgCl_2$ treatment the lung blocks were subjected to the Cuprolinic Blue staining procedure (see below).

Cuprolinic Blue staining

Lung specimens were fixed (overnight, room temperature) in 0.025 M sodium acetate buffer (*p*H 5.6), containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue (CB) (BDH-chemicals, Poole, U.K.) and 0.15 to 0.30 M $MgCl_2$. When the solution became turbid, it was renewed. After CB staining the tissue was washed (3 times 10 min) in the staining solution without CB, washed again (3 times 10 min) in aq. dest. containing 1% sodium tungstate, and dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% sodium tungstate. Embedding was in Epon [21]. In order to select alveoli for electron microscopical examination, semithin sections (1 µm) were stained with toluidin blue and basic fuchsine [2] and studied light microscopically. Ultrathin sections were collected on formvar or parlodion coated grids. When necessary, poststaining was performed with lead citrate [26] and uranyl acetate; this is indicated in the legend to the figures. Sections were examined in a Philips 201 electron microscope.

Results

Common cell types in the wall of an alveolus (Fig. 1) are epithelial cells, endothelial cells and fibroblasts. There are three types of epithelial cells: the squamous type I cell, making up about 93% of the total epithelial surface [3], the

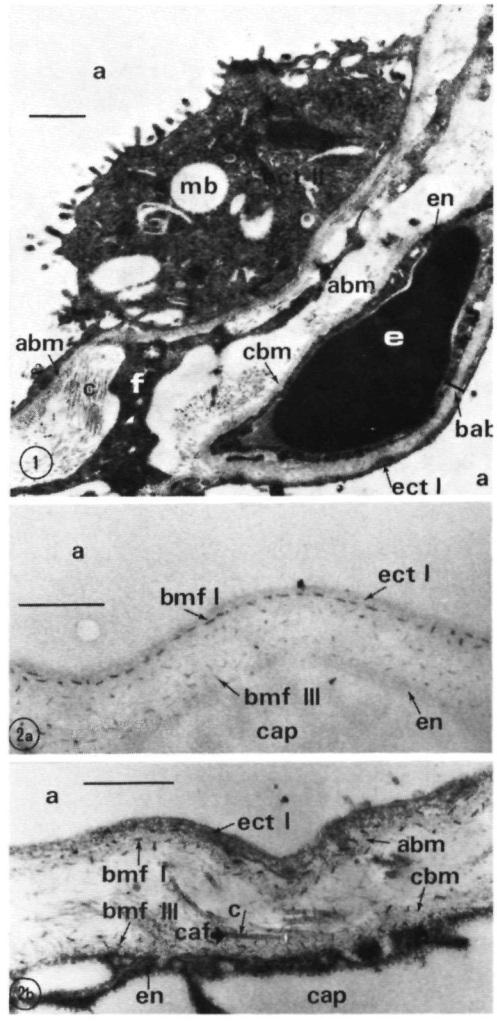


Fig. 1. Part of an alveolar wall. The most occurring cell types are type I epithelial cell (ect I), type II epithelial cell (ect II), endothelial cells (en) and fibroblasts (f). The basement membranes of the epithelial cells (alveolar basement membrane, abm) and the endothelium (capillary basement membrane, cbm) are visible. The blood-air barrier (bab) is formed by ect I, the abm, the cbm and the en. The multilamellar bodies (mb) of ect II appear empty because the phospholipids have been washed out. — a Alveolus. — c Collagen. — e Erythrocyte. — Post-stained with uranyl acetate and lead citrate. — Bar 1 µm.

Fig. 2. Blood-air barrier after Cuprolinic Blue (CB) staining. — **a.** CB-positive filaments (bmf I) become apparent in the basement membrane of type I epithelial cells; they appear to lie in one plane. The capillary basement membrane also contains CB-positive filaments (bmf III). — Bar 0.5 µm. — **b.** After post-staining with uranyl acetate and lead citrate the basement membranes of the type I epithelium and endothelium respectively become obvious; the bmf I and bmf III respectively can be seen associated with them. — Note the second layer of bmf I. Furthermore, CB-positive collagen associated filaments (caf) become apparent. — Bar 0.5 µm.

phospholipid producing cuboidal type II cell, accounting for about 7% of the epithelium and the very rare brush cell [22]. The epithelial and endothelial cells are accompanied by an alveolar and a capillary basement membrane respectively. The blood-air barrier, the thin layer of tissue separating the alveolar airspace from the blood, is formed by the type I epithelial cell, its basement membrane, the basement membrane of the endothelium and the endothelium itself. The two basement membranes can be in close apposition in the blood-air barrier [10]. Among the connective tissue elements, collagen and elastin are conspicuous.

Cuprolinic Blue staining

After Cuprolinic Blue (CB) staining various electron-dense filaments become evident. The basement membrane of both type I (Figs. 2a, 2b) and type II epithelial cells contain CB-positive filaments. They appear to lie in one plane and their length is mostly within the range of 40 to 60 nm. A dependance of the length of the filaments on the $MgCl_2$ concentration, as noticed in mouse lung alveoli [33], could not be observed. Sometimes a second layer of basement membrane filaments becomes obvious (Figs. 2b, 7a), further away from the epithelial cell membrane; its filaments are somewhat shorter and form a less continuous layer. A second layer is not present in mouse lung alveoli [33]. The capillary basement membrane also contains CB-positive filaments (Figs. 2a, 2b); they are, however, more scattered and less electron-dense when compared to the alveolar basement membrane filaments (Fig. 2a).

The collagen fibrils are also associated with CB-positive filaments (Fig. 2b). When post-staining is omitted (Fig. 3), the collagen fibrils are no longer visible, but the filaments can be easily detected. They lie, highly ordered, perpendicular with respect to the fibril long axis and are separated from each other according to the main banding period of the collagen fibrils (±60 nm); their length is about 40 nm. When compared to the alveolar basement membrane filaments they are clearly less electron-dense (Fig. 3). Filaments lying parallel to the fibril axis can also be seen (Fig. 3), as well as filaments connecting two fibrils. Finally, there is a large, heavy staining type of CB-positive filament (Fig. 4). These filaments are only locally present and are

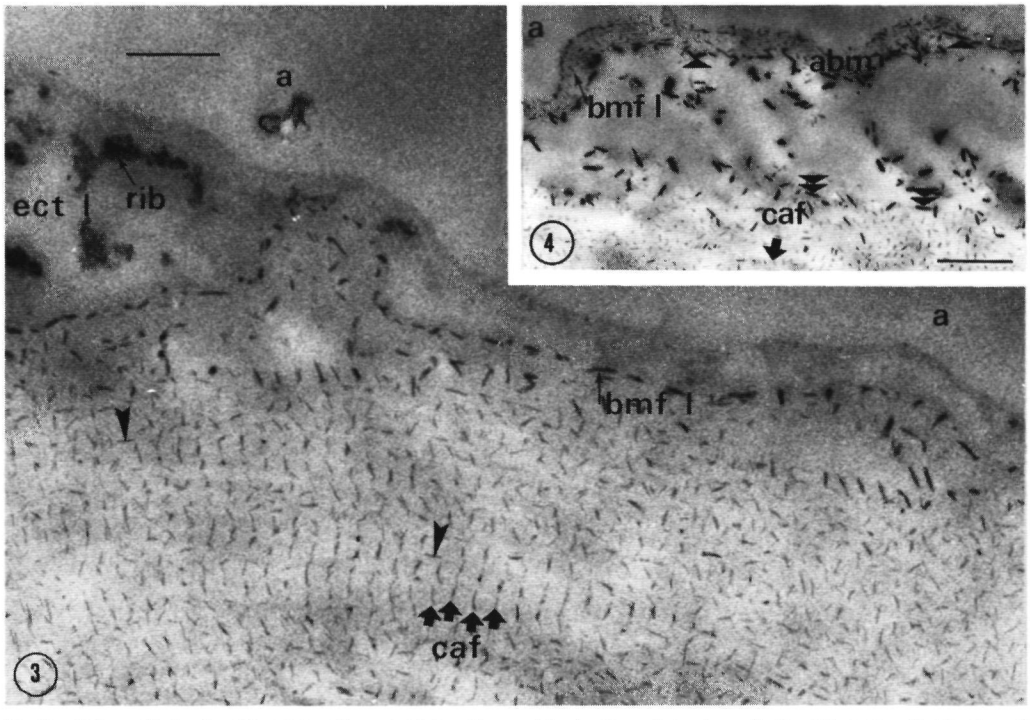


Fig. 3. Collagen fibrils after CB staining. No post-staining. No collagen fibrils can be detected; the caf, however, remain clearly visible; they are separated according to the main banding period of the fibrils (±60 nm). — Note caf lying parallel with respect to the fibril axis *(arrows)*. When compared to the bmf I the caf are less electron-dense. — rib Ribosomes. — Bar 0.25 μm.

Fig. 4. Part of alveolar wall after CB staining. Besides the caf and bmf I, large, heavy staining filaments are present; they can be seen at the boundary of collagen fibrils *(double triangles)* and associated with the alveolar basement membrane *(single triangles)*. The epithelial cell layer has been stripped off. — Bar 0.5 μm.

seen, among others, at the boundary of bundles of collagen fibrils and associated with basement membranes

Without CB no filaments are visible Due to the specific interaction of C B with nucleic acids [28], intracellular ribosomes stain (Fig 3)

Characterization of the CB-positive filaments

The C B-positive filaments localized in the alveolar and capillary basement membranes are no longer visible after treatment with crude heparinase, nitrous acid (Fig 5) or heparitinase (Fig 6) On the other hand, digestion with Streptomyces hyaluronidase, neuaminidase, chondroitinase ABC (Fig 7a) or chondroitinase AC have no effect Treatment with pronase (after prefixation) or 2 0 M $MgCl_2$ (with or without prefixation, Fig 10) has no influence either, after digestion with pronase without prefixation, however, no filaments can be detected

The stainability of the collagen associated filaments disappears after digestion with crude heparinase, chondroitinase ABC (Fig 7b) or pronase without prefixation (Fig 9) Digestion with nitrous acid, heparitinase or Streptomyces hyaluronidase has no influence, as is the case for treatment with neuraminidase, pronase (after prefixation) or 2 0 M $MgCl_2$ (after prefixation) Furthermore, chondroitinase AC has hardly, if at all, any effect on the filaments (Fig 8) After extraction with 2 0 M $MgCl_2$, without prefixation, the filaments are partially lost (Fig 10), 0 2 M $MgCl_2$, without prefixation, however, has no influence

A summary of the effects of the various treatments on the basement membrane and collagen associated filaments is given in Table I

Discussion

After CB staining five types of CB-positive filaments become visible in the human alveolar wall Three types of filaments are localized in the basement membrane of type I and type II epithelial cells respectively and of the endothelial cells, another type is associated with collagen fibrils in a regular manner and finally there is a large heavy staining type of filament, which is locally present at a variety of places Since the cationic dye CB is used under such conditions (0 15-0 30 M $MgCl_2$) that only sulphate groups and nucleic acids stain [28], it is likely that the extracellular filaments contain sulphate groups There is no contribution of sulphated glycoproteins to the stainability of the filaments since a complete disappearance of the contrast of the filaments is obtained following certain, specific, GAG degrading procedures

Basement membrane filaments

The specific susceptibility of the filaments to some GAG degrading enzymes and to nitrous acid (Tab I) makes it obvious that heparan sulphate (HS) is the only sulphated GAG present The stainability of the filaments disappears after pronase digestion, but not when this is preceded by glutaraldehyde fixation HS is therefore associated with protein, probably in a covalent manner, since treatment with 2 0 M $MgCl_2$ has no effect The basement membrane filaments thus represent proteoglycans that contain HS (HSPGs), each filament probably being a PG monomer since this is also the case in mouse lung alveoli [33] The presence of HSPGs in basement membranes has been shown for a variety of tissues [14, 18, 19, 31, 32] A proteoglycan with HS as the only GAG has been isolated from the gas exchange tissue from bovine lung [25], the basement membrane filaments might be the electron microscopical visualization of such a PG The insensitivity of the HSPGs to extraction with 2 0 M $MgCl_2$ (Fig 10) suggests that they are firmly anchored in the basement membrane, studies by others [12, 25] support this

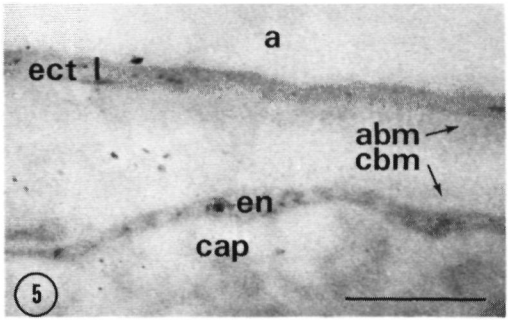
In comparison with the HSPG-filaments in the alveolar basement membrane of mouse alveoli [33], those described here are in general shorter this might reflect a larger molecular weight for the protein core of the former, since the filaments are thought to be formed by the collapse of the GAG chains on the protein core [29, 33]

The alveolar basement membrane HSPGs appear to lie closely together in one plane (Fig 2a) sometimes a second

Tab I Effects of various treatments on the basement membrane filaments type I, II and III (bmf I, II and III respectively) and on the collagen fibril associated filaments (caf)

Treatment	Substrate*	Effect on bmf I	bmf II	bmf III	caf
Neuraminidase	sialoglycoproteins	–	–	–	–
Crude heparinase	all GAGs except keratan sulphate	+	+	+	+
Streptomyces hyaluronidase	hyaluronic acid	–		–	–
Nitrous acid	heparin and heparan sulphate	+	+	+	–
Heparitinase	heparan sulphate	+	+	+	–
Chondroitinase ABC	chondroitin sulphate and dermatan sulphate	–	–	–	+
Chondroitinase AC	hyaluronic acid chondroitin sulphate and dermatan sulphate where it contains a glucuronic acid residue	–	–	–	–
Pronase (after prefixation)	proteins	–	–	–	–
Pronase (without prefixation)	proteins	+	+	+	+
2 0 M $MgCl_2$ (after prefixation)	ionic bonds	–	–	–	–
2 0 M $MgCl_2$ (without prefixation)	ionic bonds	–	–	–*	±
0 2 M $MgCl_2$ (without prefixation)	weak ionic bonds	–	–	–	–

* The specificity of the GAG degrading enzymes has been checked electrophoretically on cellulose acetate strips using copper acetate as buffer [42] — – No effect — + Filaments no longer visible — ± Filaments partially lost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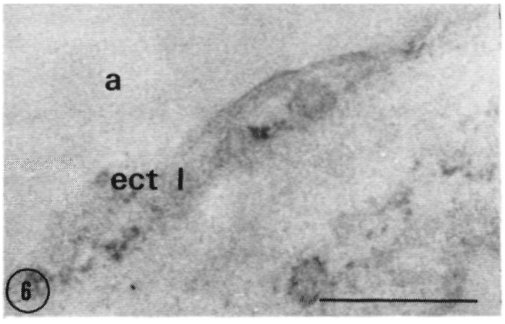
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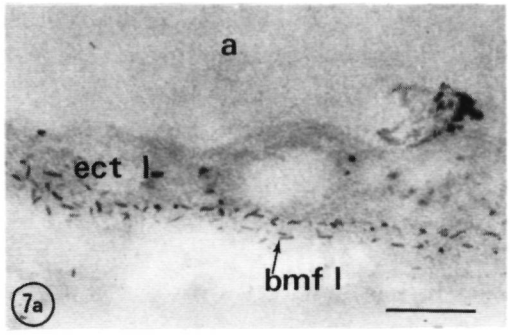
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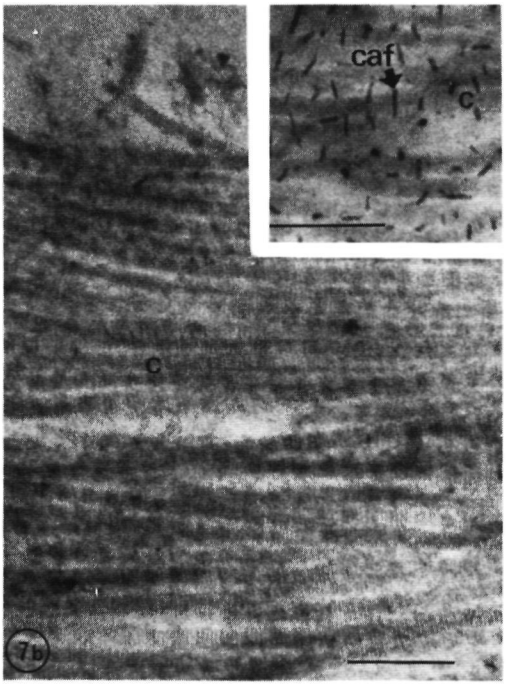
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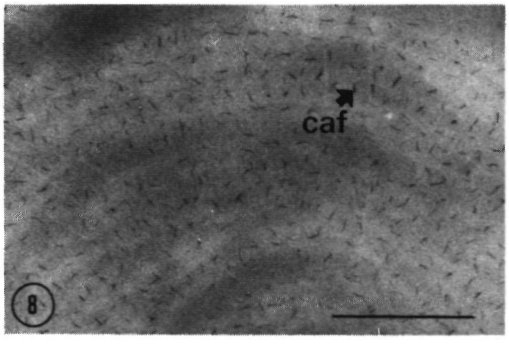
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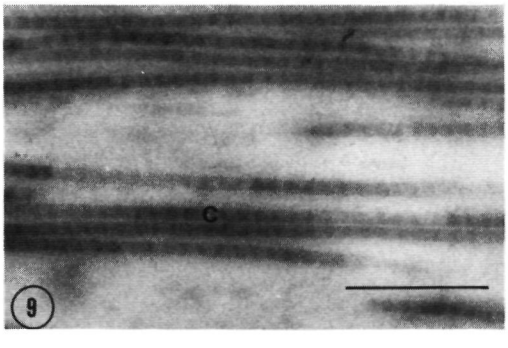
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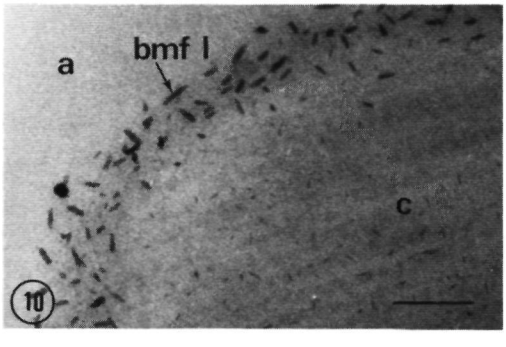
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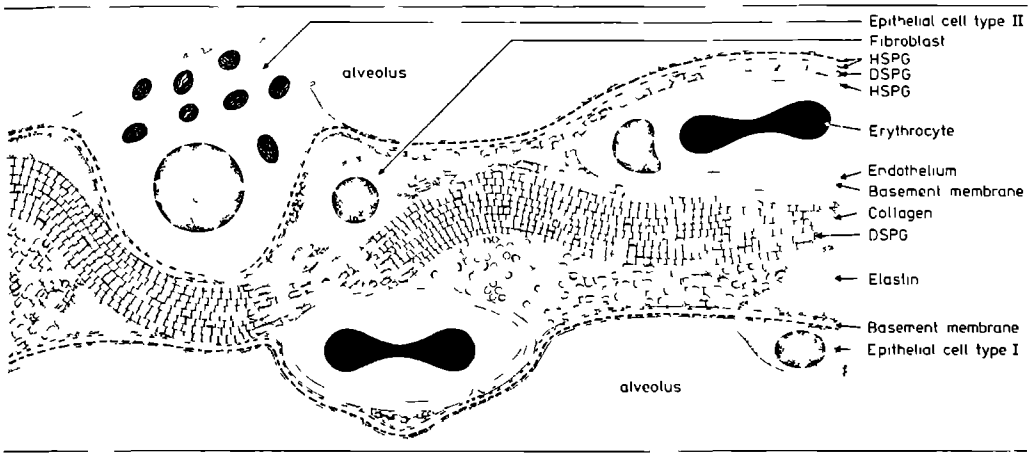

Fig. 11. Schematic representation of the localization of the various proteoglycans in human lung alveoli — HSPG Heparan sulphate containing proteoglycan DSPG Dermatan sulphate containing proteoglycan — Note the second HSPG layer, localized in the alveolar basement membrane and facing the interstitium might not always be as continuous as drawn here

layer becomes obvious The whole alveolus, therefore, contains at least two envelopes of these strongly negatively charged molecules The possible role of these envelopes in regulating the diameter of the alveolus and in influencing transport of biomolecules (filtration) has been outlined previously [34] The more scattered appearance of the HSPGs in the capillary basement membrane together with their being less electron dense (Fig 2a), may indicate different types of HSPGs in the two basement membranes The large range in sulphate content of HS chains from lung [4, 20], might explain the difference in electron density Differences in HSPGs may contribute to the heterogeneity of the alveolar and capillary basement membranes [10]

Collagen associated filaments

From the sensitivity towards various enzymatic and chemical treatments (Tab I), the filaments can be identified as proteoglycans that contain dermatan sulphate (DSPGs), the dermatan sulphate (DS) being rich in iduronic acid residues DSPGs have been isolated from a variety of tissues such as skin and tendon [5, 23, 35] and they are characterized by only few (1 4) DS chains The DSPG being less electron-dense in comparison with the HSPG of the alveolar basement membrane (Fig 3) might be due to this limited number of GAG chains in the DSPG The collagen associated PG of mouse alveoli is chondroitinase AC sensitive [34], in contrast, with that described here This reflects the presence of chondroitin sulphate and/or more glucuronic acid residues containing DS in the former It should be noted that the mice were young adults, while the lung specimens used here were from elderly patients (average age 58 years) Scott [30] noticed that during maturation the collagen associated GAGs shifted from chondroitin sulphate + hyaluronic acid to DS A similar effect might appear in humans during lifetime

The DSPGs are separated from each other according to the main banding period of the collagen fibrils (Fig 3), DSPGs connecting collagen fibrils could also be detected It seems that the collagen fibrils are surrounded by these PGs, that they are, as it were, embedded in a DSPG matrix Iduronic acid-rich DS has been shown to bind specifically to collagenous fibres in a culture system [6]

Laros and co workers [15, 16] have stressed the importance of the GAGs (PGs) for the mechanical stability of human lung parenchyma We can now, at least in part, exacerbate this to the DSPG, by connecting collagen fibrils with each other the DSPG may contribute to the lying in register of the fibrils and to achieve their functioning together as fibres, rather than as separate fibrils In such a way they may provide structural coherence as already pointed out in greater detail for mouse lung alveoli [34] Qualitative and/or quantitative alterations of the DSPGs

Fig 5 Part of alveolar wall after nitrous acid treatment The bmf I and bmf III are no longer visible Bar 0 5 µm

Fig 6. Part of alveolar wall after digestion with heparitinase The bmf I cannot be detected anymore — Bar 0 5 µm

Fig 7 Part of alveolar wall after treatment with chondroitinase ABC **a.** The bmf I are not influenced Note the double layer of bmf I — Bar 0 25 µm — **b** The caf can no longer be detected Post-stained with uranyl acetate and lead citrate Bar 0 25 µm — c Control The caf are clearly visible Post stained with uranyl acetate and lead citrate — Bar 0 25 µm

Fig 8 Part of alveolar wall after treatment with chondroitinase AC The caf are hardly, if at all, affected Bar 0 5 µm

Fig 9. Collagen fibrils after treatment with pronase without prefixation The caf are no longer visible Post stained with uranyl acetate and lead citrate — Bar 0 5 µm

Fig. 10 Part of alveolar wall after treatment with 2 0 M $MgCl_2$ without prefixation The bmf I remain unaffected, but the caf are partially lost Bar 0 25 µm

may cause a malfunction of the collagen fibrils, thereby leading to pathological conditions

Because of the varying presence of the large, heavy staining filaments and because the method of identification used in this study are not optimal for these structures, their nature remains to be established

A representation of the localization of the different PGs in human lung alveoli is given in Figure 11

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CHAPTER 6

Isolation and characterization of a collagen fibril-associated dermatan sulphate proteoglycan from bovine lung.

TOIN H.M.S.M. VAN KUPPEVELT, HENRIET M.J. JANSSEN, HENK M. VAN BEUNINGEN, KIN-SUN CHEUNG, MARTIN M.A. SCHIJEN, CHARLES M.A. KUYPER AND JACQUES H. VEERKAMP

Submitted.

Isolation and characterization of a collagen fibril-associated dermatan sulphate proteoglycan from bovine lung

Abstract

Dermatan sulphate proteoglycans have been extracted from bovine lung with 2.0 M $CaCl_2$ and isolated using CsCl density gradient centrifugation, DEAE ion exchange chromatography, gel chromatography and preparative SDS-polyacrylamide gel electrophoresis. Ultrastructurally these proteoglycans are specifically associated with collagen fibrils. Dermatan sulphate (M_r $15 \cdot 10^3$ - $35 \cdot 10^3$, with a strong prevalence for the higher M_r) is linked via an O-glycosidic bond to a protein core, which is rich in Asx, Glx and Leu. Of the total uronic acid, 91% is iduronic acid. A part of the glucuronic acid residues is located near the protein core and a large cluster of disaccharides is devoid of glucuronic acid residues.

An inhibition enzyme immuno assay has been developed to quantitate the proteoglycan; 50% inhibition occures at about 150 ng dermatan sulphate proteoglycan/ml sample.

A model for the interaction between dermatan sulphate proteoglycans and collagen fibrils is proposed.

Introduction

During breathing the lung undergoes a constant change in volume. It might be expected that this characteristic calls for special demands upon the

pulmonary connective tissue skeleton. This applies especially to lung alveoli, the structural elements in which the gas exchange process takes place. They have to possess several "opposite" characteristics in order to function efficiently. Their walls have to be 1) thin, in order to obtain a proper gas exchange; 2) firm, in order to prevent the alveolus from a collapse; 3) flexible, in order to cope with the volume change during breathing.

The three main components of the extracellular matrix of the lung connective tissue are elastin, collagen and PGs[2]. PGs can be classified according to the type(s) of glycosaminoglycans they possess. The type of glycosaminoglycan seems to be related to the function of the PG (Poole, 1986). For example heparan sulphate PGs, located in the glomerular basement membrane, play a role as selective barrier (Kanwar et al., 1980); the huge chondroitin sulphate/keratan sulphate PG of cartilage is involved in absorbing compressive load (Hascall and Hascall, 1981), while DSPGs are thought to play a role in the functioning of collagen fibrils (Van Kuppevelt et al., 1985). Dermatan sulphate PGs have been isolated from a variety of collagenous tissues such as skin (Miyamoto and Nagase, 1980), sclera (Cöster and Fransson, 1981) and tendon (Anderson, 1975; Vogel and Heinegard, 1985). These studies, however, do not deal with the ultrastructural localization of the proteoglycan. We here wish to report the isolation and characterization of a DSPG from bovine lung as well as the ultrastructural localization of this PG, using the critical electrolyte concentration method (Scott, 1980).

[2]The abbreviations used are: PG, proteoglycan; DSPG, dermatan sulphate proteoglycan; DS, dermatan sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS: 0.05 M Tris/HCl + 0.85% NaCl pH 7.5; TBST: TBS + 0.1% Tween 20; BSA: bovine serum albumin

Materials

Bovine lung, obtained from a local slaughterhouse, was put on ice, transported and used directly. Acrylamide, N, N'-methylenebisacrylamide, benzamidine HCl, 6-amino-n-caproic acid, CsCl, Alcian Blue 8GX and guanidine HCl were all from Janssen Chimica (Beerse, Belgium). Before use the guanidine HCl was treated with activated charcoal until the A_{280} (room temperature, 1 cm light-path) was below 0.05.

Chondroitinase ABC (EC 4.2.2.4), chondroitinase AC (EC 4.2.2.5), phenylmethylsulphonyl fluoride, iodoactamide, chondroitin-4-sulphate from whale cartilage, hyaluronic acid from human umbilical cord, dermatan sulphate from porcine skin, bovine serum albumin (fraction V) and fibronectin (from human plasma), were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dermatan sulphate from bovine lung was a kind gift from Dr. J.A. Cifonelli, University of Chicago, IL, U.S.A.. Toluidine Blue, Coomassie Blue R250, 1,9-dimethylmethylene Blue, disodium EDTA and Tween 20 (polyoxyethylenesorbitanmonolaurate) were from Merck-Schuchardt Darmstadt, F.R.G.). Basic fuchsine was from U.C.B., Brussels, Belgium. Papain (EC 3.4.22.2) was from Worthington (Freehold, NJ, U.S.A.). SDS and marker proteins for SDS-polyacrylamide gel electrophoresis were obtained from Serva (Heidelberg, F.R.G.). DEAE-Sephacel, Sephadex G25 (coarse) Sephadex G75, and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). Cellulose polyacetate electrophoresis was carried out on 25.5 x 14.5 cm cellulose polyacetate strips (Schleicher & Schüll, Dassel, F.R.G.) in a semimicroelectrophoresis chamber obtained from Gelman Instrument Company (Ann Arbor, MI, U.S.A.). Protamin. HCl was from Kabi AB, Stockholm, Sweden. Polystyrene microtiter plates were from Costar, Cambridge, MA, U.S.A.; polyvinyl microtiter plates were obtained from Linbro, Flow Lab., Inc. Mclean, VA, U.S.A.

Peroxidase conjugated goat anti-rabbit igG (gamma + light Ch. Sp., purified by affinity chromatography) was from Tago, Inc., Burlingame, CA, U.S.A. Rabbit peroxidase-anti-peroxidase was obtained from American Qualex Internat., Inc., La Mirada, CA, U.S.A.

Laminin was a kind gift from Dr. R. Timpl, Max Planck Institut für Biochemie, Martinsried, F.R.G. Chondroitin sulphate/keratan sulphate PG was a kind gift from Dr. R.J. van de Stadt, Jan van Breemen Institute, Amsterdam, The Netherlands.

Type I, III, IV and V collagen were isolated from human placenta by limited pepsin digestion and salt precipitation (Miller & Rhodes, 1982, Abedin et al., 1981); stock solutions were made containg 1 mg collagen/ml 0.5 M HAc.

Methods

Isolation of DSPGs

During the isolation, all steps were carried out at 4°C, unless stated otherwise. The following protease inhibitors were present up to DEAE-Sephacel chromatography: 0.1 M 6-amino-n-caproic acid, 5 mM benzamidine HCl, 1 mM phenylmethylsulphonyl fluoride, 10 mM disodium EDTA and 1 mM iodoacetamide. Lung tissue, devoid of cartilage, pleura and great vessels was grinded with a meat-grinder, degassed (in order to obtain a good penetration of the solutions) and centrifuged (10 min, 1500 g). After rinsing the tissue overnight in deionized water, centrifugation (45 min, 20,000 g), rinsing overnight again in 0.15 M NaCl and centrifugation (45 min, 20,000 g), the DSPGs were extracted for 3 h with 3 vol. of a $CaCl_2$ solution, the final concentration being 2.0 M $CaCl_2$. After centrifugation (30 min, 20,000 g), the supernatant was dialyzed versus deionized water and the resulting precipitate, after centrifugation at 20,000 g for 45 min, dissolved in 7 vol. 4 M guanidine HCl. CsCl density gradient centrifugation (Beckman SW-40 rotor, ρo = 1.48 g/ml, 100,000 g, 24 h) of this material resulted in the appearance of a gel-like substance floating at the top of the gradient. These "gels" were then pulverized in a chilled

mortar and carefully suspended in 10 vol. 2.0 M $CaCl_2$ in a potterdevice; the suspension was left overnight under continuous stirring. After centrifugation (30 min, 20,000 g), precipitation of the proteoglycans was achieved by mixing the supernatant with 3 vol. of ethanol saturated with sodium acetate and allowing it to stand for 5 h at -20°C. After centrifugation (30 min, 20,000 g) the precipitate was washed once with 80% ethanol (2 h, -20°C), centrifuged (30 min, 20,000 g) and dissolved in 50 vol. 0.05 M Tris/HCl pH 6.8 containing 7 M urea and 0.30 M NaCl. Next, this solution was mixed with one vol. of filtered DEAE-Sephacel, previously equilibrated with 0.05 M Tris/HCl (pH 6.8) containing 7 M urea (starting buffer) and stirred for 2 h . Subsequently, the slurry was washed until no protein could be detected in the buffer (assayed with A_{280}). After mixing with 1 vol. of starting buffer containing 0.5 M NaCl for 1 h., the slurry was washed with the same solution. The fractions were assayed via the Farndale microassay (see below), and those containing the DSPGs were collected. At this point two different procedures were followed:

I. The collected fractions were pooled and CsCl was added until a ρo of 1.45 g/ml was reached, and CsCl density gradient centrifugation was carried out (48 h, 100,000 g). The bottom fraction (1/5 of the total volume) was then chromatographed on a Sephadex G-75 column (40 cm x 2.5 cm) equilibrated with deionized water; the material eluting near the void volume, containing the purified DSPG, was collected and lyophylized.

II. The collected fractions were pooled and gel chromatography on Sephadex G-75 was performed as described above. After lyophylizing, about 10 mg of the material was subjected to SDS-PAGE (see below). The gel was washed overnight in 45% methanol containing 5.5% acetic acid and the DSPG was extracted from the gel using a electrophoretic sample concentrator (Model 1750, ISCO, Lincoln; NE, U.S.A.). The extracted material was subjected to gel chromatography on Sephadex G-25, which had been equilibrated with deionized water. The void volume was lyophylized.

Extraction and identification of glycosaminoglycans

Glycosaminoglycans were extracted according to a modified method of Hoffman (1968). Lung specimens were kept in aceton for 24 h at -20°C, dried in an exsiccator and (after grinding) further defatted in ether/methanol (1:1) for 48 h (4°C). All other samples were used directly. Samples were suspended in 40 vol. of 0.75 M NaOH containing 10 mM $NaBH_4$ and allowed to stand for 1 h at 73°C followed by chilling and neutralizing with 10 M HCl. In one case 0.1 M NaOH/10 mM $NaBH_4$ was used at 4°C, overnight. Ice-cold 20% trichloroacetic acid was then added to a final concentration of 5%. After 1 h on ice and centrifugation (15 min, 2000 g) the resulting pellet was washed once with 5% trichloroacetic acid and centrifuged again. The supernatants were pooled and 3 vol. of cold ethanol saturated with sodium acetate was added; after 24 h at 4°C the mixture was centrifuged (15 min, 2000 g), the pellet washed once with 80% ethanol (2 h, -20°C) and centrifuged again. The resulting pellet, representing the glycosaminoglycans, was dried and subjected to cellulose polyacetate electrophoresis according to Stevanovich and Gore (1967) using 0.1 M copper acetate (pH 3.1) as buffer. Strips were stained with 0.1% Alcian Blue in 5% acetic acid/10% ethanol and destained in 5% acetic acid.

Preparation of DS-peptides

The material obtained by precipitation with ethanol (see under isolation of DSPG) was used as starting material. At this stage DSPG is the only PG present (as determined by SDS-PAGE). After dissolving 600 mg of the ethanol precipitate in 50 ml 4 M guanidine HCl, CsCl density gradient centrifugation (ρo 1.45 g/ml, 100,000 g, 48 h) was carried out and the bottom fraction (1/5 of the total) was dialysed versus deionized water and lyophylized. Papain digestion (enzyme : substrate = 1:100) was carried out for 24 h at 65°C in 0.05 M sodium phosphate buffer (pH 7.0) containing 1 M NaCl, 0.05 M disodium

EDTA and 0.01 M cysteine HCl. After precipitation for 1 h in 10% ice-cold trichloroacetic acid and centrifugation (8000 g, 15 min), the supernatant was desalted and lyophylized. DEAE-chromatography was performed batchwise with 0.05 M Tris HCl/0.06 M NaAc pH 8.0 as starting buffer. After elution with buffer containing 0.25 M NaCl and 0.35 M NaCl the fraction eluting with 1 M NaCl was desalted and lyophylized.

Molecular weight determination of DS-peptides

DS-peptides were chromatographed on Sephadex G-200 according to Wasteson (1971). Fractions of 1 ml were collected and quantified using the Farndale microassay (see below). Lung DS (M_r of $40 \cdot 10^3$) and pig skin DS (M_r $26 \cdot 10^3$, Damle et al., 1982) were used as markers.

SDS-PAGE

SDS-PAGE was done according to Laemmli (1970). Gradient gels (6-18%) were used unless stated otherwise. Molecular weight determination of the DSPG and the protein core was done on 10% and 12.5% gels. Gels were stained with 0.25% Coomassie Brilliant Blue R-250, Toluidine Blue according to Rosenberg et al. (1985), or with periodic acid Schiff reagent according to Konat et al. (1984).

Enzymetic treatments

Digestion with chondroitinase ABC and chrondroitinase AC was performed at 37°C for 17 h in 75 µl 0.05 M Tris/HCl - 0.06 M NaAc pH 8.0 containing about 1.3 mg DSPG. Both enzymes were used at a concentration of 1,7 U/ml; in one case chondroitinase AC was applied at a concentration of 8,5 U/ml. Addition of protease inhibitors had no influence.

Analytical methods

PGs (glycosaminoglycans) were detected using the microassay which was developed by Farndale et al. (1982) and is based on a metachromatic shift in absorption maximum of 1,9-dimethylmethylene Blue. 100 µl Sample was mixed with 1 ml 0.0016% 1,9-dimethylmethylene Blue and the change in absorbance at 535 nm was determined. This method is not entirely specific (nucleic acids may interfere) but it provides a simple and fast way for the evaluation of separation procedures. When no interfering substances are present the assay can be used quantitatively.

The percentage iduronic acid of the total uronic acid content was assayed by measuring the amount of unsaturated glycuronosyl residues formed after chondroitinase digestion; a slightly modified procedure according to Hascall et al. (1972) was used. Briefly, DS-peptides were treated with chondroitinase ABC or chondroitinase AC and oxidated with 0.04 M $NaIO_4$ in 0.08 M H_2SO_4. After neutralizing with 2% $NaAsO_2$ in 0.5 M HCl, 0.3% thiobarbituric acid was added. After 15 min at 100°C the chromophore formed was extracted into cyclohexanon (Warren, 1959) and measured spectrophotometrically at 549 nm.

Hydroxyproline content was measured according to the method of Huszar et al. (1980). Alkaline hydrolysis was for 2 h at 120°C.

The presence of nucleic acids was evaluated by mixing the sample with an equal volume of 0.2% ethidium bromide in 0.01 M Tris HCl/1 mM disodium EDTA buffer (pH 8.0) (Maniatis et al., 1982). Samples were examined under UV illumination (254 nm). Calf thymus DNA was used as a standard.

Amino acid analysis was performed after hydrolysis under vacuum in 6 M HCl at 105°C for 24 h, using a Varian LC 5000 HPLC amino acid analyser.

Electron microscopy

The Cuprolinic Blue staining procedure, using the critical electrolyte

concentration method, as well as the enzymatic procedures have been outlined previously (Van Kuppevelt et al., 1985). Chondroitinase ABC and chondroitinase AC were used in a concentration of 2 U/ml for 1.5 h. Sections were poststained with 2% aqueous uranyl acetate.

Enzyme immunoassay

Immunization

DSPG was isolated after SDS-PAGE, electrophoretic extraction from the gel and chromatography on Sephadex G-25 (see above). After lyophilization, DSPG was dissolved in 0.85% NaCl (1 mg/ml) and mixed with an equal volume of complete Freunds adjuvant. A total of 2 ml was injected subcutaneously in New Zealand white rabbits at different places. After 4 weeks, rabbits were boostered with the same amount of antigen, but mixed with incomplete Freunds adjuvant. Two weeks thereafter the rabbits were bleeded and the serum collected.

Immunoadsorption

About 4 mg DSPG in 0.8 ml coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was incubated with 0.5 ml of CNBr-activated Sepharose 4B slurry for 16 h at 4°C. After blocking the remaining active groups with 0.2 M glycine (pH 8.0) for 16 h at 4°C, the excess of adsorbed protein was washed away by 5 cycles of coupling buffer followed by 0.1 M HAc/0.5 M NaCl (pH 4.0). The gel was washed again with coupling buffer and stored at 4°C in TBS containing 0.05% NaN_3. Immunoadsorption was accomplished by putting 0.5 ml slurry into a 1 ml column and pumping 150 µl serum (diluted in TBS containing 1% BSA) around for 90 min. (room temperature). The column was washed with TBS and the bounded antibodies were eluted with 1 ml 1.0 M HAc. The eluens was immediately neutralized with 4 M Tris. After addition

of BSA to a final concentration of 1%, the material was dialysed versus TBS.

Nonequilibrium inhibition enzyme immunoassay

Wells of polystyrene microtiter plates were coated with protein core obtained by overnight digestion of DSPGs (1 mg/ml 0.05 M Tris/HCl + 0.06 M NaAc, pH 8.0) with chondroitinase ABC (0.1 U/ml) at 37°C. After dilution in TBS, each well was incubated (overnight, 4°C) with 100 µl containing an amount of protein core derived from 1 µg DSPG (1 µg DSPG equivalences). After incubation the wells were treated with 200 µl TBS containing 1% BSA for 1.5 h at room temperature and washed (3 times) with TBST.

In the wells of round-bottomed polyvinyl microtiter plates, 50 µl of an antibody solution (either serum or purified antibodies were used) was added to 100 µl of the sample to be tested; dilutions were made in TBST containing 1% BSA. After incubation overnight at 4°C, 100 µl of each well was transferred to a well of a polystyrene plate previously coated with protein core. The free antibody was allowed to complex with the coated antigen for 30 min (nonequilibrium condition, Rennard et al., 1980). After washing three times with TBST, the antibodies bound to the protein core were detected by incubation (1.5 h, room temperature) with goat anti-rabbit IgG conjugated with peroxidase (1:1000 in TBST containing 1% BSA). After washing, 100 µl of a peroxidase-anti-peroxidase complex (1:500 in TBST containing 1% BSA) was added and incubation was for 1.5 h at room temperature followed by washing. Subsequently 100 µl of enzyme substrate (0.04% o-phenylenediamine and 0.016% H_2O_2 in 24 mM citric acid/51 mM Na_2HPO_4) was added and the reaction was allowed to proceed for 30 min at room temperature and stopped by addition of 100 µl 4 M H_2SO_4. The colour developed was spectrophotometrically measured at 492 nm using a Titertek^R multiscan.

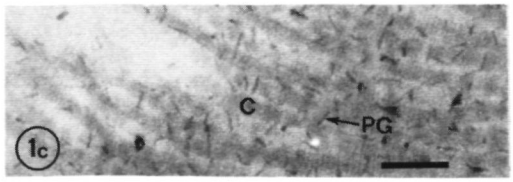
Direct enzyme immunoassay

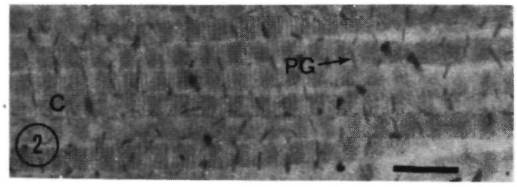
In this assay, serum is added directly to the coated polystyrene microtiter plates. Before coating with DSPG, papaine-digested DSPG, DS and calf thymus DNA, wells were pre-incubated for 2 h at room temperature, with 100 µl TBS containing 50 µg protamine HCl (Faaber et al., 1984). Papaine treatment of DSPGs was done according to Cöster and Fransson (1981). Collagens, fibronectin and laminin were diluted in 0.02 M carbonate buffer, pH 9.6 (Rennard et al., 1980) to a final concentration of 10 µg/ml. All other coating substances were diluted in TBS to a final concentration of 10 µg/ml, except for DNA (25 µg/ml). Wells were coated with 100 µl overnight at 4°C. After blocking with TBS containing 1% BSA and washing 100 µl of serum diluted 1:1000-1:9000 with TBST containing 1% BSA, was added. Incubation with serum, goat anti-rabbit IgG conjugated with peroxidase, and peroxidase-anti-peroxidase complex (see above) was either for 30 min at 37°C (Rosenberg et al., 1984) or 1.5 h at room temperature. Detection of peroxidase was as described above. As a control, pre-immune serum instead of immune serum was used.

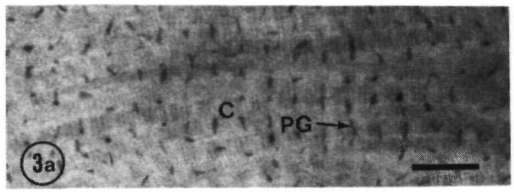
Results

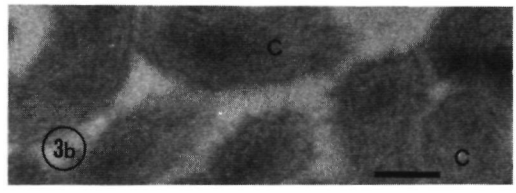
Ultrastructural localization of DSPGs.

PGs appear as electron-dense filaments after the Cuprolinic Blue staining procedure (Van Kuppevelt et al., 1985). The PGs associated with collagen fibrils from alveolar septa (Fig. 1a) are regularly arranged and separated from each other according to the main banding pattern of these collagen fibrils (about 60 nm). Most PGs are located perpendicular with respect to the collagen fibril axis although some have a parallel orientation. The collagen fibril-associated PGs are sensitive to chondroitinase ABC (Fig.









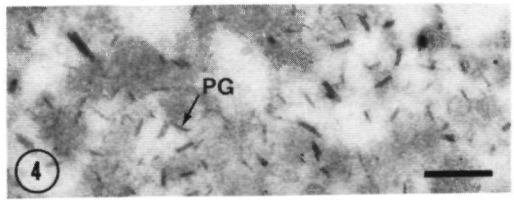


Fig. 1. Collagenous area in a bovine alveolus after the Cuprolinic Blue staining procedure. Poststained with uranyl acetate. Bar: 150 nm.
a) Proteoglycans (PGs) are associated with collagen fibrils (c) and are separated from each other according to the main banding period (about 60 nm) of the collagen fibrils. Note PGs (big arrows) lying parallel with respect to the collagen fibril axis.
b) After digestion with chondroitinase ABC, no proteoglycans can be observed.
c) After treatment with chondroitinase AC, the proteoglycans appear hardly affected.

Fig. 2. Collagenous area in the wall of a small blood vessel of lung after the Cuprolinic Blue staining procedure. Poststained with uranyl acetate. Bar: 150 nm. Proteoglycans (PGs) are present similar to those associated with alveolar collagen fibrils.

Fig. 3. Collagenous area of lung after some purification steps. The Cuprolinic Blue staining procedure has been applied. Poststained with uranyl acetate. Bar: 150 nm.
a) Collagenous area after grinding, degassing and rinsing in deionized water and 0.15 M NaCl. These treatments have no effect on the amount or location of the collagen fibril-associated proteoglycans (PGs).
b) Collagenous area after treating the lung tissue with 2 M $CaCl_2$. For further experimental details see the text. The collagen fibrils (c) are swollen; no proteoglycans are detectable.

Fig. 4. Gel-like material obtained after CsCl density gradient centrifugation of the material extracted with 2 M $CaCl_2$. For further details see the text. Stained according to the Cuprolinic Blue procedure. Poststained with uranyl acetate. Bar: 150 nm.
Proteoglycans (PGs) resembling those associated with collagen fibrils are abundant.

1b) while chondroitinase AC has hardly any effect (Fig. 1c); this indicates that they represent DSPGs. PGs associated with collagen fibrils from the wall of small bloodvessels (Fig. 2) and bronchioli are similar to those of alveoli with respect to their orientation as well as their sensitivity towards the glycosidases. The PGs are predominately located at the d-band of the collagen fibrils and in general 2-3 perpendicularly orientated PGs are present for each D-period.

Isolation of DSPGs.

The starting lung material contains hyaluronic acid, heparan sulphate, dermatan sulphate , chondroitin sulphate and some heparin (Fig. 5a). Grinding, degassing and rinsing in deionized water and in 0.15 M NaCl have no influence on the location or amount of DSPGs associated with collagen fibrils (Fig. 3a); hyaluronic acid is partially extracted by these treatments.

Subsequent treatment with 2 M $CaCl_2$ results in the extraction of the DSPGs ; the collagen fibrils remaining behind appear to be swollen (Fig. 3b). Dialysis of the 2 M $CaCl_2$ extract versus deionized water results in a precipitate which, after CsCl density gradient centrifugation, reveals a gel-like substance (ρ = 1.26 g/ml) floating at the top of the gradient. This "gel" contains DS as the predominant glycosaminoglycan; small amounts of heparan sulphate and chondroitin sulphate, however, are still present. Electron microscopical observation (Fig. 4) indicates that the "gel" contains PGs resembling to those associated with collagen fibrils as observed in situ; no collagen fibrils can be detected. The Hyp content is 1.0 µg/mg gel. Partially dissolving the gel-like substance in 2 M $CaCl_2$ followed by treatment of the extract with ethanol saturated with sodium acetate results in a precipitate in which DS is the only glycosaminoglycan present (Fig. 5b). DEAE-Sephacel chromatography of this precipitate reveals one fraction positive in the Farndale assay and eluting at 0.5 M NaCl (Fig. 6). After CsCl

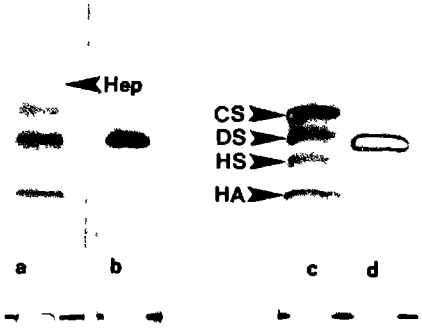


Fig. 5. Cellulose polyacetate electrophoresis of glycosaminoglycans (GAGs) extracted from material at various stages of the isolation procedure.
a) GAG-composition of the lung. Hep: heparin.
b) GAG-composition of the material extracted from the gel-like material obtained after CsCl density gradient centrifugation. For further details see the text.
c) Reference GAGs. HA: hyaluronic acid; HS: heparan sulphate; DS: dermatan sulphate; CS: chondroitin-4-sulphate. 0.5 µg of each GAG was applied.
d) GAG-composition of the isolated proteoglycan. Because an overload of GAGs was applied, the centre of the DS-band appears unstained.

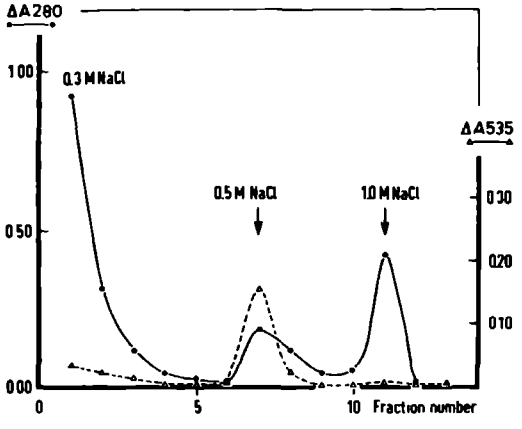


Fig. 6. DEAE-Sephacel chromatography of the material extracted with 2 M $CaCl_2$ from the gel-like material obtained after CsCl density gradient centrifugation. After precipitation with ethanol saturated with sodium acetate, the pellet was dissolved in 0.05 M Tris/HCl pH 6.8 containing 7 M urea and 0.3 M NaCl and subjected to DEAE-Sephacel chromatography. Elution was performed with starting buffer containing 0.5 M NaCl and 1.0 M NaCl, respectively. Separation was done batchwise. Protein was detected via the A_{280}; glycosaminoglycans were assayed by measuring the shift in absorption maximum (A_{535}) of 1,9-dimethylmethylene blue according to Farndale et al., (1982).

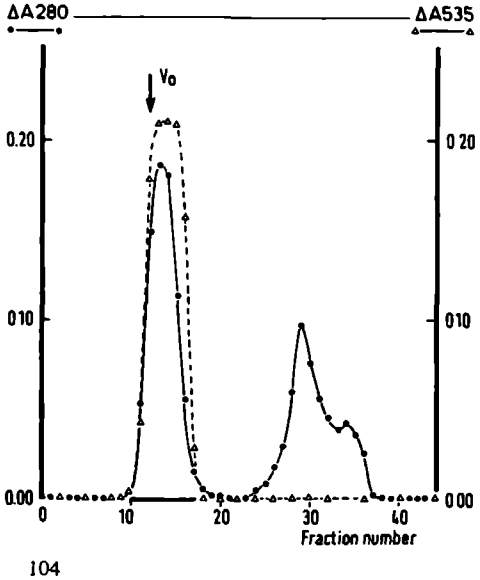


Fig. 7. Gel chromatography on Sephadex G-75 of the 0.5 M NaCl fraction obtained after DEAE-Sephacel chromatography. Elution was performed with deionized water. The presence of protein was assayed by measuring the A_{280}; glycosaminoglycan detection was done by measuring the shift in absorption maximum (A_{535}) of 1,9-dimethylmethylene blue according to Farndale et al. (1982). Each fraction represents about 5 ml.

density gradient centrifugation of this fraction, DSPG is about equally distributed along the gradient (ρ = 1.43-1.55 g/ml); the bottom fraction, however, contains only one contaminating low-molecular weight protein which can be removed by gel-chromatography on Sephadex G-75. The peak eluting near the void volume contains the purified DSPG. DSPG is obtained in higher yield, however, when the second CsCl density gradient centrifugation step is omitted. In this case, the material eluting at 0.5 M NaCl is chromatographed directly on Sephadex G-75 (Fig. 7) and the material eluting near the void volume is subjected to SDS-PAGE. DSPG is electrophoretically extracted from the gel. Only a trace of nucleic acids (about 0.1%) can be detected in this preparation.

Characterization of the DSPG.

After SDS-PAGE, the DSPG appears as a single broad Coomassie Blue-positive band, which also stains with periodic acid-Schiff reagent and metachromatically with Toluidine Blue (Fig. 8). Addition of β-mercaptoethanol has no influence. The M_r of the DSPG is $50 \cdot 10^3$-$80 \cdot 10^3$. Staining with Toluidine Blue and periodic acid-Schiff reagent also reveals a less intense, slower migrating broad band (Fig. 8), its midpoint corresponding to a M_r of $100 \cdot 10^3$. Cellulose polyacetate electrophoresis reveals that DS is the only glycosaminoglycan present (Fig. 5d); its mobility is somewhat slower when compared to DS from pig skin. Of the total uronic acid content 91% is iduronic acid; in DS from pig skin we found 86% iduronic acid, a value which is in good agreement with the literature (Damle et al., 1982). The M_r of the protein core, obtained after digestion with chondroitinase ABC, is $17 \cdot 10^3$. It should be noted that the protein core is much more Coomassie Blue-positive than the intact DSPG; the protein core did not stain with Toluidine Blue (Fig. 8). Only when a large overload was applied, the protein core faintly stained with periodic acid-Schiff reagent. In

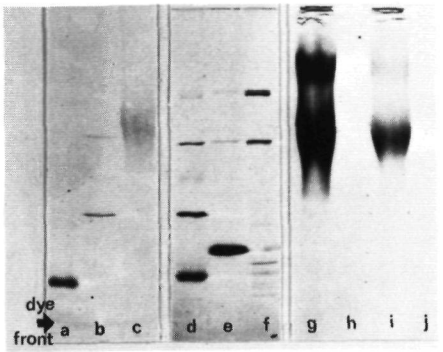


Fig. 8. SDS-polyacrylamide gel electrophoresis of the dermatan sulphate proteoglycan (DSPG) before and after treatment with chondroitinase ABC.
a-f: Coomassie Blue staining.
g,h: Toluidine Blue staining.
i,j: periodic acid Schiff reagent staining.
a) reference protein: cytochrome c (M_r $12.5 \cdot 10^3$).
b) reference proteins: bovine serum albumin (M_r $68 \cdot 10^3$), ovalbumine (only weakly visible, M_r $45 \cdot 10^3$), carbonic anhydrase (M_r $29 \cdot 10^3$).
c, g, i) DSPG.
d) reference proteins: phosphorylase B (M_r $92.5 \cdot 10^3$), bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome c.
e, h, j) DSPG after treatment with chondroitinase ABC.
f) chondroitinase ABC.

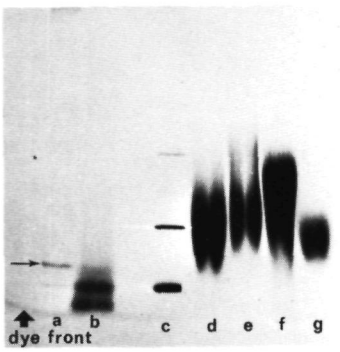


Fig. 9. SDS-polyacrylamide gel electrophoresis of the dermatan sulphate proteoglycan (DSPG) after NaOH/$NaBH_4$ treatment.
a - c: Coomassie Blue staining.
d-g: Toluidine Blue staining.
a, e) DSPG after treatment with 0.1 M NaOH/10 mM $NaBH_4$ at 4 °C, overnight. The position of the protein core is indicated.
b, d) DSPG after treatment with 0.75 M NaOH/10 mM $NaBH_4$ for 1 h at 73 °C.
c) reference proteins: bovine serum albumin (M_r $68 \cdot 10^3$), ovalbumin (only weakly visible, M_r $45 \cdot 10^3$), carbonic anhydrase (M_r $29 \cdot 10^3$) and cytochrome c (M_r $12.5 \cdot 10^3$).
f) reference DS (M_r $40 \cdot 10^3$).
g) reference DS (M_r $26 \cdot 10^3$).

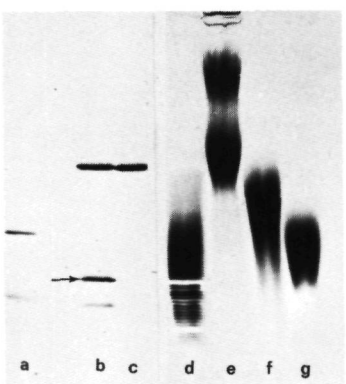


Fig. 10. SDS-polyacrylamide gel electrophoresis of the dermatan sulphate proteoglycan (DSPG) after treatment with chondroitinase AC.
a-c: Coomassie Blue staining.
d-g: Toluidine Blue staining.
a) reference proteins: carbonic anhydrase (M_r $29 \cdot 10^3$) and cytochrome c (M_r $12.5 \cdot 10^3$).
b) DSPG after chondroitinase AC treatment (1.7 U/ml). The protein core is indicated.
c) chondroitinase AC.
d) DSPG after chondroitinase AC treatment (8.5 U/ml).
e) DSPG.
f) reference DS (M_r $40 \cdot 10^3$).
g) reference DS (M_r $26 \cdot 10^3$).

case of a large overload, also a protein core with a M_r of about $45 \cdot 10^3$ becomes detectable. Amino acid analysis of the DSPG (Table I) indicates that the protein core is rich in Asx, Glx and Leu residues.

Chromatography on Sephadex G-200 indicates that the M_r of the DS-peptides ranges from $15 \cdot 10^3$-$35 \cdot 10^3$, with a strong prevalence for the higher molecular weights. SDS-PAGE of DS obtained after treatment with 0.1 M NaOH/10 mM $NaBH_4$ (Fig. 9) also shows that the majority of the DS is in the upper part of that range ($30 \cdot 10^3$-$35 \cdot 10^3$). When DSPG is subjected to treatment with 0.1 M NaOH/10 mM $NaBH_4$ at 4°C, overnight, a large part is split into the protein core and DS (Fig. 9). When digestion is performed at harder conditions (0.75 M NaOH/10 mM $NaBH_4$, 1 h, 73°C), the protein core is largely degraded while the liberated DS has a somewhat lower M_r when compared to that obtained after mild treatment (Fig. 9).
Digestion of the DSPG with chondroitinase AC (Fig. 10) results in the appearance of the protein core and DS of which a large part comigrates with pig skin DS, even when the enzyme is applied at a concentration of 8.5 U/ml. Smaller fragments, however, also occur.
Inhibition enzyme immunoassays and/or direct enzyme immunoassays demonstrate that the anti-DSPG antiserum reacts with DSPG and protein core, but not with type I, III, IV and V collagen, laminin, fibronectin and DNA (data not shown). It also does not react with papaine-digested DSPG and DS, indicating that the antibodies are raised against the protein core rather than the DS. The purified antibodies react equally well with DSPG and protein core (Fig. 11); they do not bind with a chondroitin sulphate/keratan sulphate PG from cartilage. The DSPG concentration required for 50% inhibition was about 150 ng/ml sample (Fig. 11).

Asx	116
Thr	42
Ser	69
Glx	130
Pro	90
Gly	79
Ala	61
Val	57
Cys	nd[a]
Met	9
Ile	51
Leu	128
Tyr	13
Phe	32
Lys	60
His	28
Arg	35

Table I. Amino acid composition of the dermatan sulphate proteoglycan. Values are expressed as residues per 1000 residues and are not corrected for destruction during hydrolysis. a: not determined.

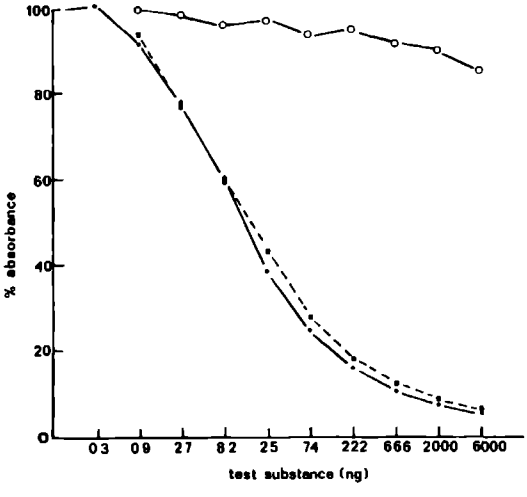


Fig. 11. Inhibition enzyme immunoassay with purified antibodies raised against dermatan sulphate proteoglycans (DSPGs).
100 µl of test substance was incubated overnight, 4°C, with purified antibodies. Next, 100 µl of this mixture was transferred to a corresponding well previously coated with an amount of protein core derived from 1000 ng DSPG after digestion with chondroitinase ABC (i.e. 1000 ng DSPG equivalences). After incubation for 30 min. at room temperature, the antibodies bound to the well were detected as described in the text. The absorbance at 429 nm is expressed as a percentage in comparison with that without the test substance, which is taken as 100%.
•——•——•: DSPG
▪- -▪- -▪: protein core as expressed in DSPG equivalences
o——o——o: chondroitin sulphate/keratan sulphate proteoglycan from cartilage.

Electron microscopical study shows that collagen fibrils in lung are associated with small proteoglycans; enzymatic digestions reveal that these proteoglycans contain dermatan sulphate rich in iduronic acid residues. Chondroitinase ABC sensitive PGs have been shown to occur in sheep lung (Sampson et al., 1979) while it has also been reported that DSPGs are present in rabbit and bovine lungs (Radhakrishnamurty et al., 1980, 1985). A major concern in the isolation of PGs is the possibility of degradation. The major DSPG isolated in this study has a M_r of $50 \cdot 10^3$-$80 \cdot 10^3$ and a protein core with a M_r of $17 \cdot 10^3$. Usually we also observed a larger DSPG. The midpoint of the broad band representing this DSPG corresponds to a M_r of about $100 \cdot 10^3$. When applying an overload, a protein core became detectable with a M_r of about $45 \cdot 10^3$. Recent information on DSPGs from the lungs of guinea pigs and rats indicate a M_r of $100 \cdot 10^3$ for the PG and a M_r of about $47 \cdot 10^3$ for the protein core (Hernnas et al., 1986). Furthermore, small DSPGs isolated from a variety of collagenous connective tissues seem to have a M_r of about $100 \cdot 10^3$ and a protein core with a M_r of $40 \cdot 10^3$-$60 \cdot 10^3$ (Heinegard et al., 1985; Hassell et al., 1986). In the light of these literature data it may be possible that the major DSPG isolated in this study is not the intact PG. Despite the presence of protease inhibitors, the protein core could have been degraded; in this case, the observed larger DSPG could be the intact DSPG with a protein core with a M_r of about $45 \cdot 10^3$.

DSPGs were extracted with 2.0 M $CaCl_2$. Although in most studies 4 M guanidine HCl is used for extraction of the DSPG, 2 M $CaCl_2$ has (in our case) the advantage of leaving most other PGs (e.g. heparan sulphate PGs from basement membranes) behind in the residue. During CsCl density gra-

dient centrifugation a gel-like substance (ρ: 1.26 g/ml) is formed containing DSPG as the predominant PG. This material contains no collagen fibrils and only little collagen (as judged by the Hyp content). In a study on cardiac valve PGs, Honda et al. (1977) mentioned a similar gel-like substance at the top of the gradient; it contained < 1% Hyp, had a ρ of 1.32 g/ml and was composed predominantely of DSPG and glycoproteins. At present we cannot explain the gel formation.

The DSPG of lung contains a very high content of iduronic acid residues compared to those of other tissues (Poole, 1986). After digestion with chondroitinase AC, which digests DS only where it contains a glucuronic acid residue, a considerable portion of the DSPGs is split into the protein core and a DS which comigrates with pig skin DS (M_r about $26 \cdot 10^3$). This indicates that a part of the glucuronic acid residues is located near the protein core as in pig skin DSPG (Fransson & Malmström, 1971). It furthermore points to the presence of a large cluster of disaccharides in the DS-chain lacking glucuronic acid residues. In an ultrastructural study on DSPGs from human alveoli, we found that chondroitinase AC has no effect when applied for 90 min at 2 U/ml (Van Kuppevelt et al., 1985); however, when digestion is performed for 16 h at 4 U/ml the DS is split off (unpublished results). A possible explanation is that the enzyme may come close to the protein core only at an elevated concentration and a prolonged incubation time. This also indicates that glucuronic acid residues are positioned near the protein core. Since DS is degraded by chondroitinase AC into fragments of various molecular weights it is likely that glucuronic acid residues also occur in other regions of the DS-chain.

After treatment with 0.1 M NaOH/10 mM $NaBH_4$, the DSPGs are split into the protein core and DS. Mild alkaline/borohydride treatment is known to split O-glycosidic bonds between protein and sugar-chain through the process of β-elimination (Stern et al., 1971; Spiro, 1972). N-Glycosidic bonds

are not affected, provided that the borohydride concentration is sufficiently low (Rasilo & Renkonen, 1981). This result therefore indicates that an O-glycosidic linkage exists between DS and protein core. Xylose and serine are involved in this linkage in pig skin DSPG (Stern et al., 1971).

Taking together the data on the DSPG, a model can be constructed in which a small protein core bears, via O-glycosidic bond(s), 1-3 DS chains rich in iduronic acid residues. The lung DSPG seems to belong to a class of DSPGs, derived from collagenous tissues, and characterized by a relatively low M_r, a small protein core rich in Leu, Asx and Glx, a relatively high protein/glycosaminoglycan ratio and one or few iduronic acid-rich DS-chains (Miyamoto & Nagase, 1980; Fujii & Nagai, 1981; Cöster & Fransson, 1981; Pearson & Gibson, 1982; Uldbjerg et al., 1983; Nakamura et al., 1983; Vogel & Heinegård, 1985). These DSPGs seem to be immunologically related to each other (Heinegård et al., 1985).

During the Cuprolinic Blue staining procedure, the glycosaminoglycan side chains collapse onto the protein core (Scott, 1980; Van Kuppevelt, 1984), thereby causing the PGs to appear as filaments rather than extended, open structures. This means that the electron-dense filaments indicate the location of the protein core. It might therefore be argued that it is the protein core that specifically binds to the collagen fibril. An interaction between collagen and the protein core of a small DSPG of tendon has been noted (Vogel et al., 1984). For each D-period there would be 2-3 DSPG molecules orientated perpendicularly with respect to the collagen fibril axis. The bond between collagen and the protein core is probably (at least partly) ionic in nature since DSPGs can be removed from the collagen fibril by treatment with 2 M $MgCl_2$ (Van Kuppevelt et al., 1984a) or 2 M $CaCl_2$. Glutaraldehyde, which fixes proteins (Cheung and Nimni, 1982), prevents removal (Van Kuppevelt et al., 1984a). The protein core is rich in Asx and Glx and may therefore contain a considerable amount of acidic amino acids. In a part of a protein core of a DSPG from bovine skin all Asx and Glx are present as Asp and Glu, respectively (Pearson et al., 1983). Such amino acids would favour an ionic bond and may bind to the basic amino acids of the d-band to

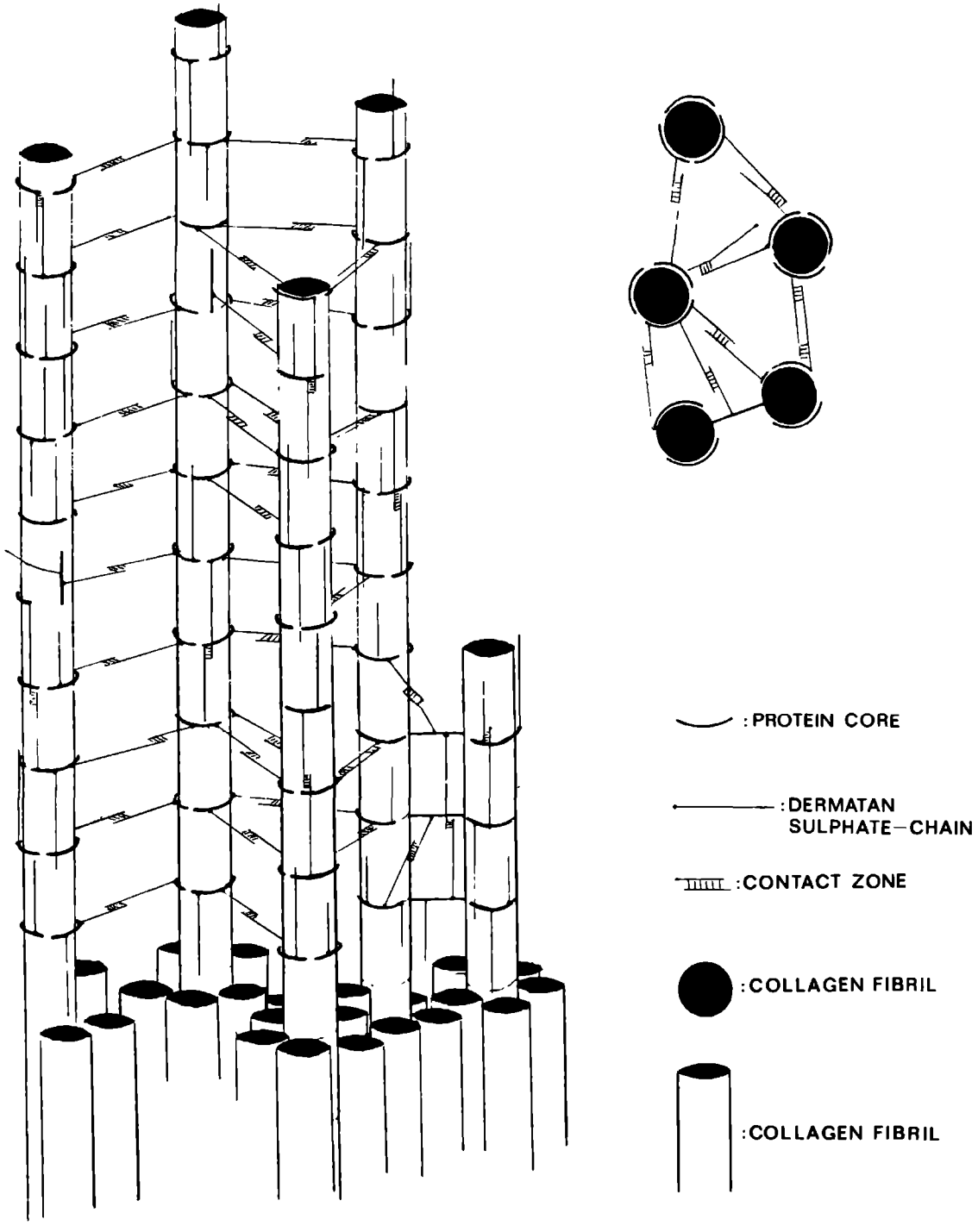


Fig. 12. Schematic model of the interaction between dermatan sulphate proteoglycans (DSPGs) and collagen fibrils. a) lateral view; b) top view).
The protein core of the DSPG binds specifically to the collagen fibrils, while the dermatan sulphate chains bind to each other. In addition, dermatan sulphate chains binding to collagen fibrils occur, as well as protein cores interconnecting fibrils. In this was the DSPG may act as a cementing substance and link collagen fibrils together, thereby providing structural coherence.

which the protein core is predominantely associated. The question as to why it is the d-band, remains unanswered; other bands on the collagen fibril contain basic amino acids as well (Tzaphlidou et al., 1982; Chapman et al., 1981). Studies on DSPGs from bovine sclera (Fransson et al., 1982) have shown that DSPGs are capable of self-association, DS being of major importance. Taking a length of 1 nm/disaccharide (Atkins & Isaac, 1974; Hascall, 1980), a DS-chain of 35 kDa would be about 80 nm, which is long enough to enable association with DS-chains from other DSPGs (e.g. located on adjacent collagen fibrils). Taking together these data we propose the following model (Fig. 12). The protein core of a DSPG is attached specifically to a collagen fibril while the DS-chain(s) is (are) associated to DS-chains of other DSPGs. Binding of DS-chains to collagen fibrils may also occur as well as protein cores connecting collagen fibrils to each other. In this way DSPGs link collagen fibrils to each other and form a stable matrix. The possible physiological function of DSPGs has been discussed elsewhere (Van Kuppevelt et al., 1984a, 1985). Whether or not fibronectin, which is also periodically distributed along a collagen fibril (Yamada, 1981; Torikata et al., 1985), plays a role in the DSPG-collagen interaction remains to be established.

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CHAPTER 7

Further characterization of a large proteoglycan in human lung alveoli.

TOIN H.M.S.M. VAN KUPPEVELT, HENK M. VAN BEUNINGEN, TWAN L.M. RUTTEN ADRIAAN J.C. VAN DEN BRULE AND CHARLES M.A. KUYPER

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Further characterization of a large proteoglycan in human lung alveoli

Toin H. M. S. M. van Kuppevelt[1)], Henk M. van Beuningen, Twan L. M. Rutten, Adriaan J. C. van den Brule, Charles M. A. Kuyper

Department of Chemical Cytology, Faculty of Science, University of Nijmegen/The Netherlands

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Proteoglycans — chondroitin sulfate — dermatan sulfate — lung alveoli — collagen — basement membrane

By use of the cationic dye Cuprolinic Blue in a critical electrolyte concentration method, heavily staining, generally large, filaments have been demonstrated in human lung alveoli. In some lung specimens they are abundant, while in others they are very scanty. The filaments are seen: 1) around bundles of collagen fibrils, 2) at places which seem electron microscopically almost empty, 3) associated with basement membranes 4) around elastin, and 5) sometimes associated with individual collagen fibrils. After poststaining tiny threads — connecting the filaments — could sometimes be observed. The filaments are resistant to treatment with nitrous acid, heparitinase or pronase after prefixation. After digestion with chondroitinase ABC, chondroitinase AC or pronase without prefixation, the filaments are no longer detectable. The tiny threads are chondroitinase ABC resistant. It is concluded that the Cuprolinic Blue-positive filaments represent proteoglycans which contain chondroitin sulfate and/or glucuronic acid-rich dermatan sulfate. The possible role of these proteoglycans in tissue repair is discussed.

Introduction

Proteoglycan monomers can be visualized at the electron microscopical level by using the cationic dye Cuprolinic Blue according to the critical electrolyte concentration method [14]. In such a way we have localized in human lung alveoli heparan sulfate-containing proteoglycans as well as dermatan sulfate (DS)-containing proteoglycans [19]. We furthermore reported on the presence of a large, heavily staining filament which, however, could not be characterized at that time. In this paper we now wish to describe the ultrastructural localization and characterization of this Cuprolinic Blue-positive filament.

Materials and methods

Human lung specimens were obtained as described previously [19].

[1)] Drs. Toin H. M. S. M. van Kuppevelt, Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen/The Netherlands.

Enzyme and nitrous acid treatment

Due to the large amount of staining material bound to the Cuprolinic Blue-positive filaments, the incubation times and/or enzyme (nitrous acid) concentrations used before [19], had to be increased in most cases. Table I gives the incubation times and the enzyme (nitrous acid) concentrations applied in this study; the substrate(s) sensitive to these treatments are indicated as well. Other parameters (e.g. *p*H, temperature, buffer) remained the same [19]. In all

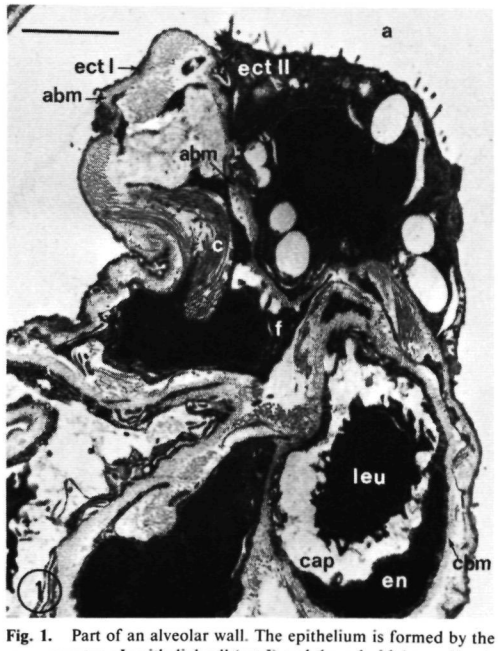


Fig. 1. Part of an alveolar wall. The epithelium is formed by the squamous type I epithelial cell (ect I) and the cuboidal type II epithelial cell (ect II), both associated with an alveolar basement membrane (abm). Furthermore, endothelial cells (en) with their capillary basement membrane (cbm) and fibroblasts (f) are present. — cap Capillary. — c Collagen fibrils. — leu Leucocyte. — a Alveolar airspace. — Post-stained with uranyl acetate and lead citrate. — Bar 2 µm.

cases, except pronase, the enzyme or nitrous acid treatment is preceded by prefixation with 2.5% glutaraldehyde in 0.025 M sodium acetate buffer (*p*H 5.6) for 2 h (4 °C). After the enzyme or nitrous acid digestion the lung specimens are subjected to the Cuprolinic Blue staining procedure.

Cuprolinic Blue staining procedure

The Cuprolinic Blue staining method has been outlined previously [19]. Briefly, the lung blocks are fixed overnight (room temperature) in 0.025 M sodium acetate buffer (*p*H 5.6) containing 2.5% glutaraldehyde, 0.2% Cuprolinic Blue and 0.2 M $MgCl_2$. After washing in buffer without Cuprolinic Blue, the specimens are rinsed in aq. dest. containing 1% sodium tungstate and dehydrated in ascending concentrations of ethanol where the 30 and 50% concentrations contain 1% sodium tungstate. The tissue is embedded in Epon. Ultrathin sections are examined in a Philips 201 electron microscope. When necessary, poststaining with uranyl acetate (with or without lead citrate) is applied.

Results

Figure 1 depicts the cell types and their extracellular products commonly found in human alveoli. Among them are type I and type II epithelial cells and their alveolar basement membranes, endothelial cells with their capillary basement membrane and fibroblasts. In the extracellular matrix the collagen fibrils are obvious. As described previously [19], heparan sulfate-containing proteoglycans are localized in the basement membrane while DS-containing proteoglycans, rich in iduronic acid residues, are associated with collagen fibrils. We also made mention of a large, heavily staining Cuprolinic Blue-positive filament; this will now be studied further.

Ultrastructural localization of the heavily staining filaments (hsf)

In general, the hsf are large (upto 200 nm), but smaller forms exist. In some lung blocks they are abundant, while in others they are very scanty. One of the main places at which they appear is around bundles of collagen fibrils (Figs. 2a, b), although they may also be associated with individual fibrils (Fig. 3). Furthermore, they can be seen in association with basement membranes (mainly at the interstitial side, Fig. 4) and in places which seem electron microscopically almost empty (Fig. 5). To a lesser extent the hsf appear associated with elastin (Fig. 6). At places where type II epithelial cells transform into type I epithelial cells they are often associated with the basement membrane (Fig. 7). Sometimes the hsf are connected with each other by very tiny threads (Fig. 2a), which are visible only after poststaining.

Characterization of the hsf

The main difficulty in characterizing the hsf lies in their capricious occurrence, only lung specimens in which they were abundant were used for studying their chemical nature. After treatment with nitrous acid (2 or 24 h, Fig. 8), heparitinase or pronase following prefixation the stainability of the hsf is unaffected. Digestion with chondroitinase ABC (Fig. 9) or chondroitinase AC (Fig. 10) results in a disappearance of this stainability, although after relative short incubation times (2 h) some remnants may be present. After digestion with pronase without prefixation no hsf can be detected. Table 1 summarizes these results. The tiny threads, connecting the hsf, appear to be chondroitinase ABC resistant, when applied during 45 min, and 1 U/ml.

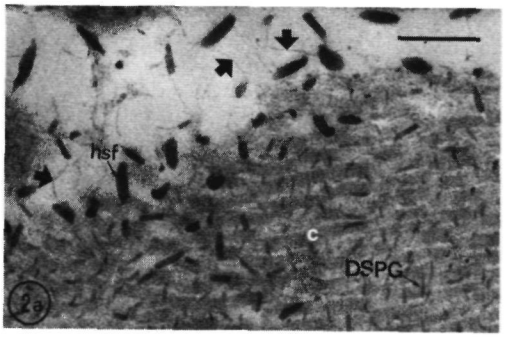
Discussion

The regular morphology of the filaments indicates a definite structure rather than an accidental cluster of smaller elements. From Table I it may be concluded that the Cuprolinic Blue-positive, heavily staining filaments represent proteoglycans (PGs). The glycosaminoglycans of these PGs are chondroitin sulfate (CS) and/or DS which is rich in glucuronic acid residues (compare the intact staining capacity of the small collagen-associated DSPG in Figure 10 after chondroitinase AC). The length of this CS/DSPG is rather large, probably reflecting a large protein core [17]; its heavy staining character points to the presence of many sulfate groups (carboxylic groups do not contribute to the contrast of the filaments because of the $MgCl_2$ present during staining [13]). In this way the CS/DSPGs resemble the well known cartilage PGs [5]. The PGs may be synthe-

Tab. I. Incubation time, concentration, substrate and the effect on the heavily staining filaments (hsf) of the enzyme and nitrous acid treatments used for characterization

Treatment	Incubation time	Concentration	Substrate(s)	Effect on hsf
Chondroitinase ABC	90 min	2 U/ml	chondroitin sulfate and	+*
	16 h	4 U/ml	dermatan sulfate	+
Chondroitinase AC	90 min	2 U/ml	chondroitin sulfate	+*
	16 h	4 U/ml	hyaluronic acid and dermatan sulfate where it contains a glucuronic acid residue	+
Heparitinase	2 h	10 U/ml	heparan sulfate	–
Nitrous acid	2 h		heparan sulfate and	–
	24 h[b]		heparin	–
Pronase (after prefixation)	45 min	15 U/ml	proteins	–
	2 h			
Pronase (without prefixation)	45 min	15 U/ml	proteins	+
	2 h			

No effect — + Filaments no longer visible — +* Sometimes remnants are present — [b] The nitrous acid solution was renewed three times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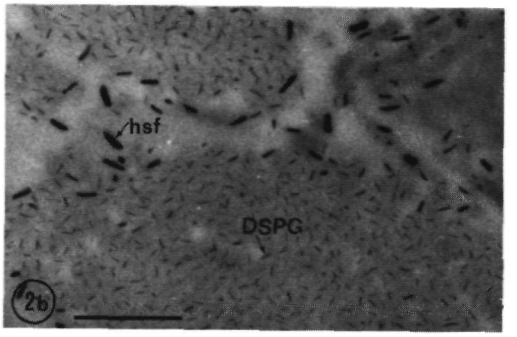
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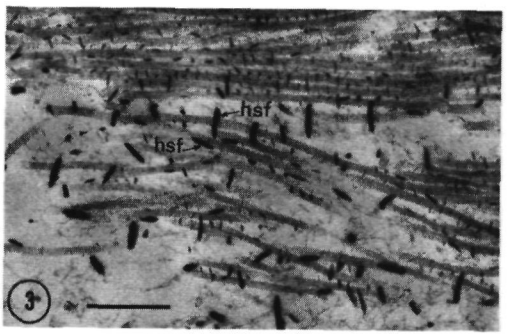
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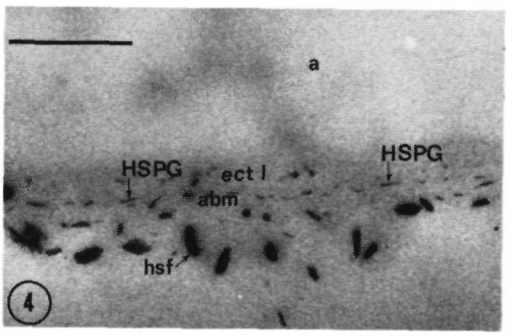
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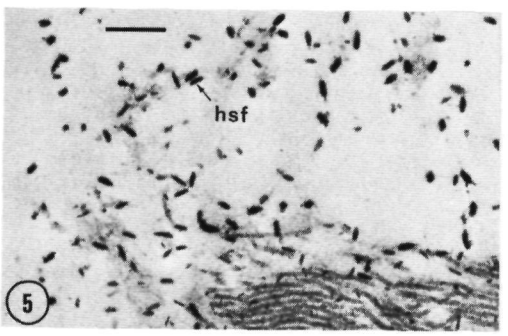
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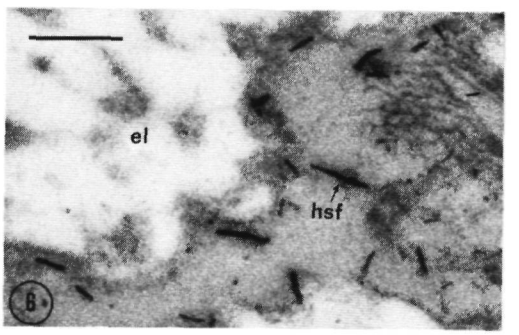
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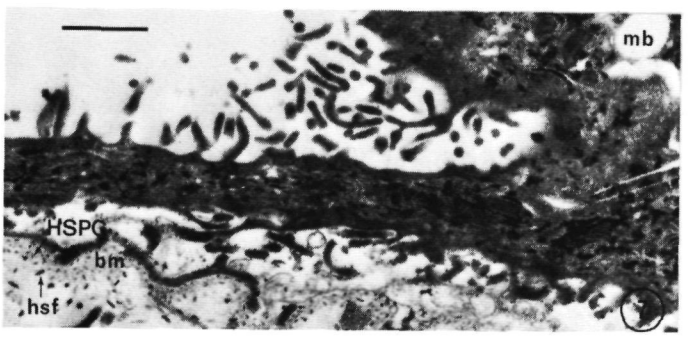
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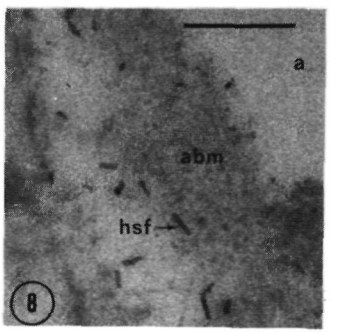
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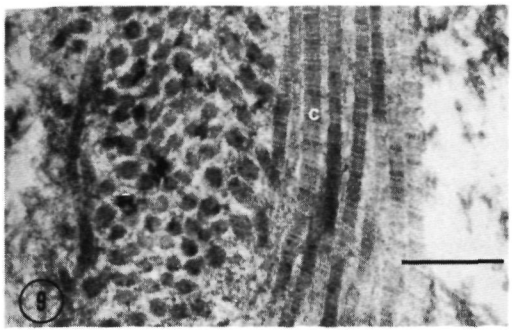

Fig. 9. Collagenous area after digestion with chondroitinase ABC for 90 min. The hsf as well as the dermatan sulfate-containing proteoglycans are no longer visible. — Post-stained with uranyl acetate and lead citrate. — Bar 0.25 μm.

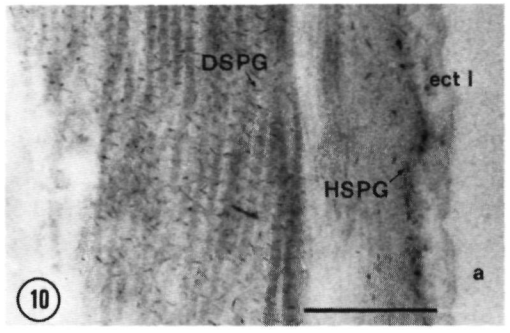

Fig. 10. Collagenous area after digestion with chondroitinase AC for 90 min. The hsf are no longer detectable; the dermatan sulfate-containing proteoglycans are not affected. — Post-stained with uranyl acetate and lead citrate. — Bar 0.5 μm.

sized by fibroblasts; it has been shown [4] that human skin fibroblasts in culture produce DS rich in iduronic acid as well as in glucuronic acid. The thread-like structures, connecting the PGs do not contain sulfate groups, as they are only contrasted by post-staining. Corresponding structures have been observed in embryonic cornea [8] and aorta [5]; the filaments in the cornea were hyaluronidase sensitive.

Fig. 2. Collagen-rich area in alveolar wall after Cuprolinic Blue staining. — **a.** Part of a bundle of collagen fibrils surrounded by heavily staining filaments (hsf). Tiny threads *(arrows)* sometimes arise from the hsf. — Note the dermatan sulfate-containing proteoglycans (DSPG) associated with collagen fibrils. — Post-stained with uranyl acetate and lead citrate. — Bar 0.25 μm. — **b.** Collagen bundle surrounded by hsf. Because the post-staining has been omitted, the fibrils themselves are not visible; the DSPG, however, mark their localization. — Bar 0.5 μm.

Fig. 3. Heavily staining filaments (hsf) associated with individual collagen fibrils. In general, the hsf lie perpendicular or parallel in respect to the collagen fibril long axis. — Post-stained with uranyl acetate and lead citrate. — Bar 0.5 μm.

Fig. 4. Heavily staining filaments (hsf) associated with the interstitial side of the alveolar basement membrane (abm). — HSPG Heparan sulfate-containing proteoglycan. — a Alveolar airspace. — Bar 0.5 μm.

Fig. 5. Heavily staining filaments (hsf) localized in areas which seem electron microscopically almost empty. — Post-stained with uranyl acetate and lead citrate. — Bar 0.5 μm.

Fig. 6. Heavily staining filaments (hsf) associated with elastin (el). — Post-stained with uranyl acetate and lead citrate. — Bar 0.25 μm.

Fig. 7. Heavily staining filaments (hsf) associated with the basement membrane (bm) of a type I epithelial cell being formed out of a type II epithelial cell, pointing to repair processes. — mb Multilamellar body. — HSPG Heparan sulfate-containing proteoglycan. — Post-stained with uranyl acetate and lead citrate. — Bar 1 μm.

Fig. 8. Alveolar basement membrane (abm) after treatment with nitrous acid for 24 h. The hsf remain unaffected; the alveolar basement membrane-associated heparan sulfate-containing proteoglycans are no longer detectable. The epithelial cell layer is no longer present. — Post-stained with uranyl acetate. — Bar 0.25 μm.

The most striking feature of the CS/DSPGs is their irregular presence. Obviously, they are involved in local processes in human lung tissue. One of such processes is tissue repair. It might be expected that in older lungs (the lung specimens used in this study were from elderly persons, average age 63 years) destruction and repair phenomena are more common than in young lungs. In this respect it is noteworthy that the CS/DSPGs were not observed in the lungs of young, adult mice [17, 18]. Furthermore, the appearance of type II epithelial cells transforming into type I epithelial cells (Fig. 7) points to alveolar cell renewal [3].

An increase in hyaluronic acid and/or CS/DS has been observed in various healing tissues such as healing tendon [12], regenerating liver [2], open skin wounds [1], healing myocardial infarction [15] and regenerating ear cartilage [7]. Interestingly, Hassell and coworkers [6] noticed the appearance of an unusually large CS/DSPG in regenerating cornea. This PG, with an increase in sulforylation of the glycosaminoglycan-chains, disappeared when transparency of the cornea was restored. Hay [8] observed large PG-granula in opaque embryonic cornea in contrast to small granula in the more mature cornea.

The localization of the CS/DSPG may further strengthen the suggestion of their participation in tissue repair processes. Basement membranes are important structures in cell attachment; they also play a prominent role in promoting cell differentiation and morphogenesis [9]. Cell movement often takes place in highly hydrated matrices [16]; the CS/DSPGs localized in areas which seem electron microscopically almost empty and consequently are very hydrated (although some material might have been extracted during tissue preparation) may be involved in creating and maintaining such matrices. The polyanionic character of the CS/DSPG is very suited for retaining large amounts of water; it is known that PGs from cartilage occupy large hydrodynamic volumes [5]. At the same time, the CS/DSPGs localized around collagen bundles may protect the collagen fibrils from an attack by proteases by forming a strongly negatively charged shield.

Studies on hypertrophic scars in vitro [11] indicate that CS(PG) can prevent collagenase from breaking down collagen; corneal PGs (but not keratan sulfate) are able to inhibit collagen lysis by collagenase (in vitro) for nearly 100% [10]

In the light of the points mentioned above it would be interesting to look for the CS/DSPGs in an experimental or natural wound healing system in the lung

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CHAPTER 8

Ultrastructural localization and characterization of a large proteoglycan involved in mouse lung development.

TWAN L.M. RUTTEN, TOIN H.M.S.M. VAN KUPPEVELT, HENRIET M.J. JANSSEN AND CHARLES M.A. KUYPER

Submitted.

Abstract

By use of the cationic dye Cuprolinic Blue in a critical electrolyte concentration method, the lungs of mice ranging from the late fetal stage (17 days of gestation) to the puberal stage (27 days) were surveyed for their proteoglycans. An unusually large proteoglycan is present within the connective tissue of lungs of late fetal and young postnatal mice. This large proteoglycan is mostly located at the boundary between large extracellular matrix structures and electron microscopically empty areas, but sometimes also at the surface of fibroblast-like cells. The proteoglycan appears to be most abundantly around 2 days postnatally. From day 10 on, its number decreases dramatically, and it can be no longer observed in the lungs of 27 day old mice. The large proteoglycan is no longer detectable after digestion with chondroitinase ABC or chondroitinase AC, but it is resistant to treatment with nitrous acid. It is therefore concluded that this large proteoglycan contains chondroitin sulfate and/or dermatan sulfate rich in glucuronic acid. Cellulose polyacetate electrophoresis of extracted glycosaminoglycans reveals that hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate are present in all stages, and that the glycosaminoglycan composition changes considerably during mouse lung development. The possible role of this proteoglycan in cell attachment and in cell migration phenomena in the developing mouse lung is discussed. Based on literature data and our own results, it is proposed that large proteoglycans containing chondroitin sulfate and/or dermatan sulfate rich in glucuronic acid, may be a common feature of developing and healing tissues.

Introduction

In our studies on proteoglycans (PGs) in lung alveoli we use the cationic dye Cuprolinic Blue according to the critical electrolyte concentration method, in order to visualize proteoglycans at the electron microscopical level. We recently reported on the presence of a large, heavily staining chondroitin sulfate and/or glucuronic acid-rich dermatan sulfate proteoglycan (CS/DS PG) in human lung alveoli (Van Kuppevelt et al., 1985b). Ascribing a role to this proteoglycan was difficult because both its presence and location in the alveolar interstitium is variable. Since lung samples were from persons with an average age of 58 years, an age at which destruction of alveolar tissue becomes a common phenomenon (Thurlbeck, 1967; Yamanaka, 1970), we assumed that this proteoglycan might be involved in lung tissue repair processes.

Several studies have indicated that healing tissues show a remarkable increase in chondroitin sulfate content, compared with normal-tissue counterparts (Edward et al., 1980; Reid and Flint, 1974; Shetlar et al., 1978). This elevated content in chrondroitin sulfate is also known for developing tissues (Horwitz and Crystal, 1975; Radhakrishnamurthy and Berenson, 1980; Sampaio and Dietrich, 1981). Chondroitin sulfate content diminishes as the repair or developmental process is nearing its completion (Edward et al., 1980; Horwitz and Crystal, 1975; Kawamoto and Nagai, 1976; Sampaio et al., 1977).

The hypothesis underlying the present study is that the chondroitin sulfate present in healing and developing tissues manifests itself, at least in part, in the shape of the large proteoglycan described above, and that this proteoglycan may be a characteristic component of such tissues. To obtain evidence for this hypothesis we examined developing mouse lungs in which this kind of proteoglycan should be present. Several

stages of lung development ranging from the late fetal (17 days of gestation) to the puberal (27 days old) lung were used. These stages cover a period in which mouse lung morphology changes most dramatically (Ten Have-Opbroek, 1981). In earlier studies we did not find a large CS/DS PG in the lungs of young adult mice (Van Kuppevelt, 1984a,b).

Materials and methods

Tissue

Lungs of K-strain (inbred) mice were used. In order to obtain lungs from late fetal mice, 17 day pregnant mice were sacrificed (expected term at day 20, the moment of appearance of the vaginal plug was taken as t=0). The embryos were dissected and their lungs removed. Young mice, 2, 6, 10, 13 and 27 days old, were killed and their lungs removed. For Cuprolinic Blue staining, enzyme and nitrous acid treatment, small lung blocks ($\leq$ 1 mm^3) were cut and degassed in the first solution they were placed in.

Chemicals

Cuprolinic Blue was obtained from BDH Chemicals Ltd, Poole, England; chrondroitinase AC (from Proteus vulgaris), chondroitinase ABC (from Arthrobacter aurescens), chondroitin-4-sulfate (from whale cartilage), hyaluronic acid (from umbilical cord) and dermatan sulfate (from porcine skin), were obtained from Sigma Chemical Company, St. Louis, USA. Heparan sulfate (from bovine lung) was a kind gift from prof. J.A. Cifonelli, University of Chicago, Illinois, USA.

Cuprolinic Blue staining

The Cuprolinic Blue staining procedure was performed as outlined previously (Van Kuppevelt et al., 1985a). Occasionally lung specimens were postfixed after the Cuprolinic Blue staining procedure, with 1% aqueous osmium tetroxide for 2 hr.

Enzyme and nitrous acid treatment

Except for the lung samples from 10 day old mice, lung samples from all ages were subjected to enzyme and nitrous acid treatment. Before these treatments,

lung specimens were prefixed for 2 hr (4°C) in 25 mM sodium acetate buffer (pH 5.6) containing 2.5% glutaraldehyde. For late fetal lung this prefixation was insufficient to prevent a wash out of extracellular matrix (ECM) components during the subsequent enzyme and nitrous acid digestion. Therefore late fetal and 2 day old mouse lung specimens were incubated overnight (4°C) in 25 mM sodium acetate buffer (pH 5.6) containing 4% glutaraldehyde. After prefixation the lung blocks were rinsed once for 10 min (4°C) in the same buffer without glutaraldehyde. The conditions of the enzyme and nitrous acid treatments (e.g. pH, temperature and buffer) were as described earlier (Van Kuppevelt et al., 1985a). In Table I the incubation time and concentrations of the enzymes and nitrous acid used are given, together with the substrates sensitive to these treatments. After the enzyme and nitrous acid digestions the lung blocks were stained with Cuprolinic Blue, dehydrated and embedded in Epon (Van Kuppevelt et al., 1985a).

Light and electron microscopy

The morphological development of the mouse lung was studied light microscopically. For this purpose semithin sections (1 µm) were cut on a Sorvall MT 5000 microtome, and stained with Toluidine Blue and Basic Fuchsine. Electron microscopy was carried out on ultrathin sections, collected on parlodion and coal coated grids, and poststained with 3% aqueous uranyl acetate for 8-15 min. The sections were examined in a Philips 201 electron microscope.

Extraction and separation of glycosaminoglycans (GAGs)

GAGs were extracted according to a modified method of Hoffman (1968). Lungs were kept in aceton for 24 hr at -20°C, dried in an exsicator and (after grinding) further defatted in ether/methanol (1:1) for 48 hr (4°C).

The material was dried again, suspended in 40 vol. of 0.75 M NaOH containing 10 mM $NaBH_4$ and allowed to stand for 1 hr at 73°C, followed by chilling and neutralizing with 10 M HCl. Ice-cold 20% trichloroacetic acid was then added to a final concentration of 5% and, after 10 hr on ice and centrifugation (15 min, 2000 g), the resulting pellet was washed once with 5% trichloroacetic acid and centrifuged again. The supernatants were pooled and 3 vol. of cold ethanol saturated with NaAc was added. After 24 hr at 4°C the mixture was centrifuged (15 min, 2000 g), the pellet was washed once with 80% ethanol (2 hr, -20°C) and centrifuged again. The resulting pellet, representing the GAGs, was dried and subjected to cellulose polyacetate electrophoresis according to Stevanovish and Gore (1967) using 0.1 M $Cu(Ac)_2$ (pH 3.1) as buffer. Strips were stained with 0.1% Alcian Blue in 5% acetic acid/10% ethanol and destained in 5% acetic acid. After making the strips transparent with paraffin oil, they were scanned densitometrically with a Zeis PMQ II spectrophotometer at 600 nm.

Lung morphology. The morphological appearance of the lungs examined is shown in the Figs. 1a-5a. Development of the mouse lung seems similar to the development of the rat lung (Burri, 1974). At 17 days of gestation the airspaces are separated by very thick septa (Fig. 1a). Although the interstitium represents by far the largest part of the tissue, little ECM is present within it (Fig. 1b). Between 17 days of gestation and 2 days postnatally the airspaces are considerably dilatated, and the septa of the now called saccules have become much thinner (Fig. 2a). In the lungs of 6 day old mice the originally smooth septa are covered with bud-like structures, bulging into the airspaces of the saccules (fig. 3a). The are the onset of the formation of new secundary septa which give rise to the alveoli. The original septa are called primary septa. The 13 day old mouse lung shows a further progressed septation, alveoli are now clearly recognizable (Fig. 4a). The former saccules have become alveolar sacs. The structure of the lungs of 27 day old mice (Fig. 5a) resembles that of the adult mouse lungs. Compared with the 13 day old lung there has been a further thinning of the septa. At the electron microscopical level a distinct increase in the amount of the ECM can be observed during the period between 17 days of gestation and 27 days postnatal. Within the interstitium, fibroblast-like cells, which in the late fetal stage lie in areas with little ECM material (Fig. 1b), end up almost completely surrounded by ECM compounds at day 27 (Fig. 5b).

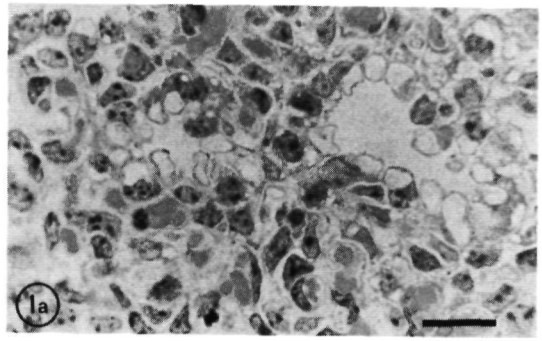
GAG composition in developing mouse lung. The relative proportions of the GAGs found in all stages of mouse lung development are presented in Fig. 12. It should be stressed that the cellulose polyacetate electrophoresis used here to separate GAGs is only semiquantitative; only major changes in GAG composition will be revealed. In mouse lung of every stage chon-

Fig. 1. Mouse lung, 17 days of gestation.
a) The late fetal mouse lung at the light microscopical level. It is a relatively compact organ; the highly cellular mesenchym is prominent. Toluidine Blue and Basic Fuchsine. Bar: 4 µm.
b) The interstitium of the late fetal mouse lung. It contains little extracellular matrix (ECM) components. Large proteoglycans (lpg) are associated with individual collagen fibrils (c), which are hardly ordered. Besides the lpg, two other two other types of proteoglycans are visible: small dermatan sulfate proteoglycans (DSPG), which are associated with collagen fibrils, and heparan sulfate proteoglycans (HSPG) in basement membranes (bm).
f: fibroblast-like cell.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 2. Mouse lung, day 2.
a) The air chambers of the 2 day old mouse are greatly enlarged compaired with the late fetal stage. The walls of these so called saccules (s) are rather smooth. Toluidine Blue and Basic Fuchsine. Bar: 7 µm.
b) Large proteoglycans (lpg) are visible around a bundle of collagen fibrils (c), and at the surface of cell processes (cp). Note that there are no lpg located within the bundle of ordered collagen fibrils.
ep: epithelial cell.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 3. Mouse lung, day 6.
a) The previously smooth walled septa have become covered with the outgrowths of small buds, bulging into the airspace of the saccules (s). The saccules are transforming into alveolar sacs. The forming secundary septa subdivide the saccules, giving rise to the alveoli (arrows). Toluidine Blue and Basic Fuchsine. Bar: 7 µm.
b) Large proteoglycans (lpg) are located at the surface of non-identified extracellular matrix structures and at the surface of a fibroblast-like cell (f). Note that there are still large areas (asterix) within the interstitium devoided of any visible structure.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 4. Mouse lung, day 13.
a) In the 13 day old mouse lung the septation has proceeded further, and alveoli (a) can be clearly distinguished. The former saccules are transformed in alveolar sacs (as). The septa are becoming thinner. Toluidine Blue and Basic Fuchsine. Bar: 7 µm.
b) The open spaces within the interstitium are largely filled up. Large proteoglycans (lpg) have become enclosed by large extracellular matrix structures.
Poststained with uranyl acetate. Bar: 0.4 µm.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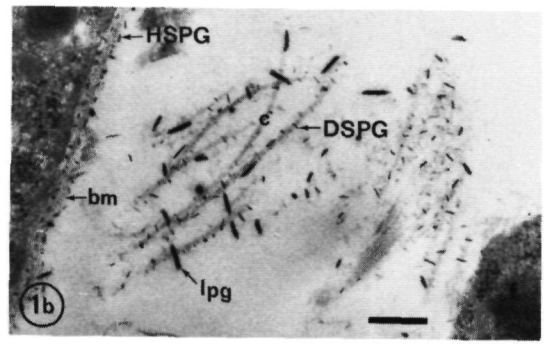
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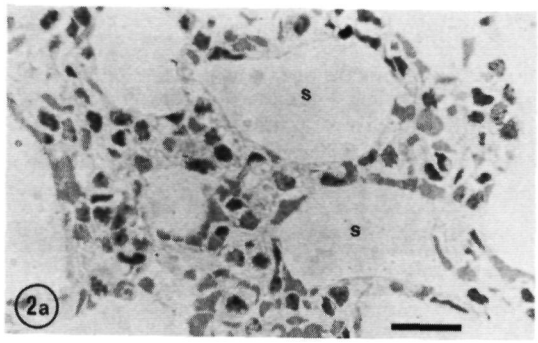
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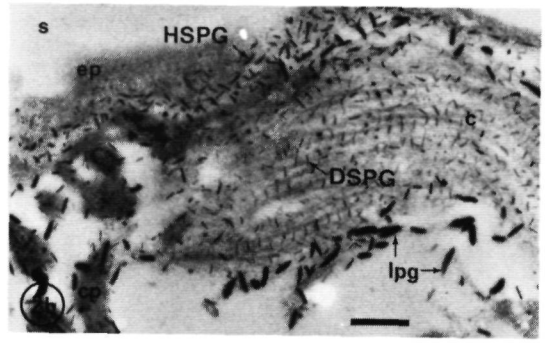
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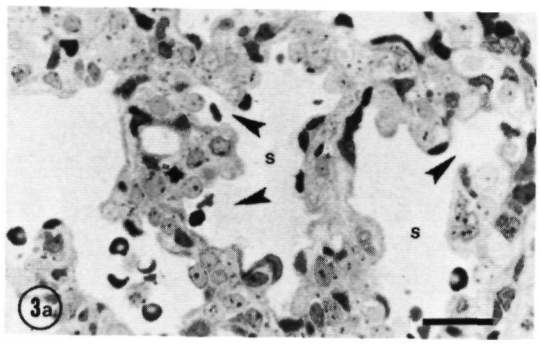
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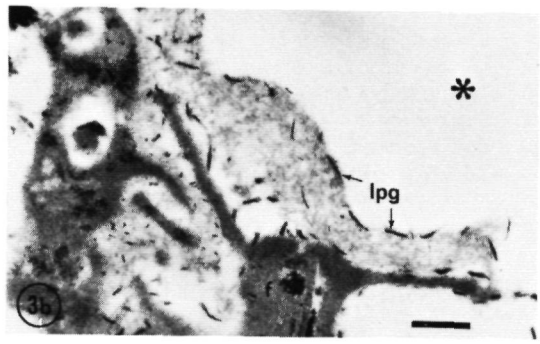
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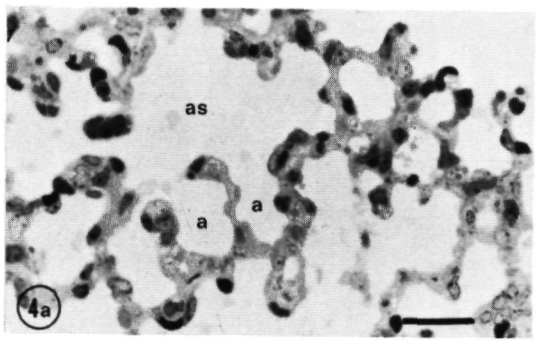
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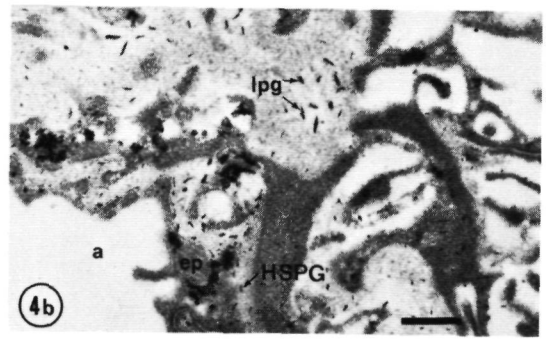
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droitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and hyaluronic acid (HA) are present. There are marked differences in the proportional distribution of the lung GAGs with development. There is a reduction of the relative amount of HA between day 2 and 6 postnatal, and of CS between day 6 and 13. The relative amount of HS increases during development; this is probably due to the extensive enlargement of the respiratory surface, and the concomitant increase of basement membranes. It has been shown that HS is an important constituent of basement membrane in rat lung (Vaccaro and Brody, 1979, 1981) and mouse lung (Van Kuppevelt et al., 1984a,b).

Presence and ultrastructural localization of PGs in developing mouse lung. The late fetal mouse lung is poorly provided with connective tissue elements; many areas are electron microscopically almost devoid of ECM structures. The possibility of losing ECM materials during tissue processing should be considered. No difference, however, can be observed between the appearance of the ECM after a normal Cuprolinic Blue staining, and after a Cuprolinic Blue staining procedure followed by a postfixation with 1% aqueous OsO_4 for 2 hr. It has been shown that addition of cationic dyes reduces the loss of proteoglycans to less than 1% (Chen and Wight, 1984). These data indicate that probably little or no wash out of ECM components occurs.

In all stages of mouse lung development we found collagen fibril - and basement membrane - associated PGs. The susceptibility of these PGs to the enzymes and nitrous acid, is identical in all stages of development (Table I). This indicates that the collagen fibril associated PGs are DSPGs while the basement membrane associated PGs are HSPGs, as is the case in adult mouse lung (Van Kuppevelt et al., 1984a,b).

Large PG

17 Days of gestation. In the late fetal mouse lung, a large heavily staining PG is quite common within the connective tissue. This large PG is often associated with individual collagen fibrils, or with amorphous, sometimes fibrillar-like, material (Fig. 1b). At this stage of mouse lung development collagen is seldom seen in compact bundles. In general the collagen fibrils are loosely organized. Whenever the collagen fibrils are assembled into tightly packed bundles, the large PG is located only at exterior of the bundles. Occasionally large PGs are located directly at the cell surface.

Day 2. The lung is crowded with the large PG. Compared with the late fetal stage their number has increased markedly (Fig. 2b). Often they are situated around bundles of collagen fibrils or associated with non-identified structures (Fig. 7). In general the large PG occurs at the boundary between ECM structures and electron microscopically empty areas. Sometimes large PGs are present on the surface of fibroblast like cells (Fig. 2b and 6). Besides the large PG, a small PG can be present on the cell surface as well. At the periphery of the lung extraordinarily large PGs can be found (Fig. 8).

Day 6. The large PG is still present in great numbers (Fig. 3b), although somewhat less abundant compared with day 2. Their ultrastructural localization is the same as in the 2 day old mouse lung.

Day 10 and 13. On day 10 the number of the large PG has fallen off sharply, while at day 13 they have become rather scarce. Over large tissue areas they have disappeared. Occasionally small numbers are left, mostly completely enclosed by ECM components (Fig. 4b). The large open spaces within the ECM, present in younger mouse lungs, are almost filled up now.

Day 27. The large PG can no longer be detected (Fig. 5b). Light microscopically, the lung resembles the adult mouse lung.

Fig. 5. Mouse lung, day 27.
a) The alveolar septa of the 27 day old mouse lung have become much thinner compared with the 13 day old mouse lung. Toluidine Blue and Basic Fuchsine. Bar: 7 µm.
b) No open spaces are left within the interstitium of the puberal mouse lung. Large proteoglycans are no longer present.
el: elastin.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 6. Mouse lung, day 2.
Large proteoglycans (lpg) are present at the surface of cell processes (cp), which are located in electron microscopically empty areas. Besides the lpg, small proteoglycans (large arrows) are visible at the surface of cell processes.
Poststained with uranyl acetate. Bar: 0.3 µm.

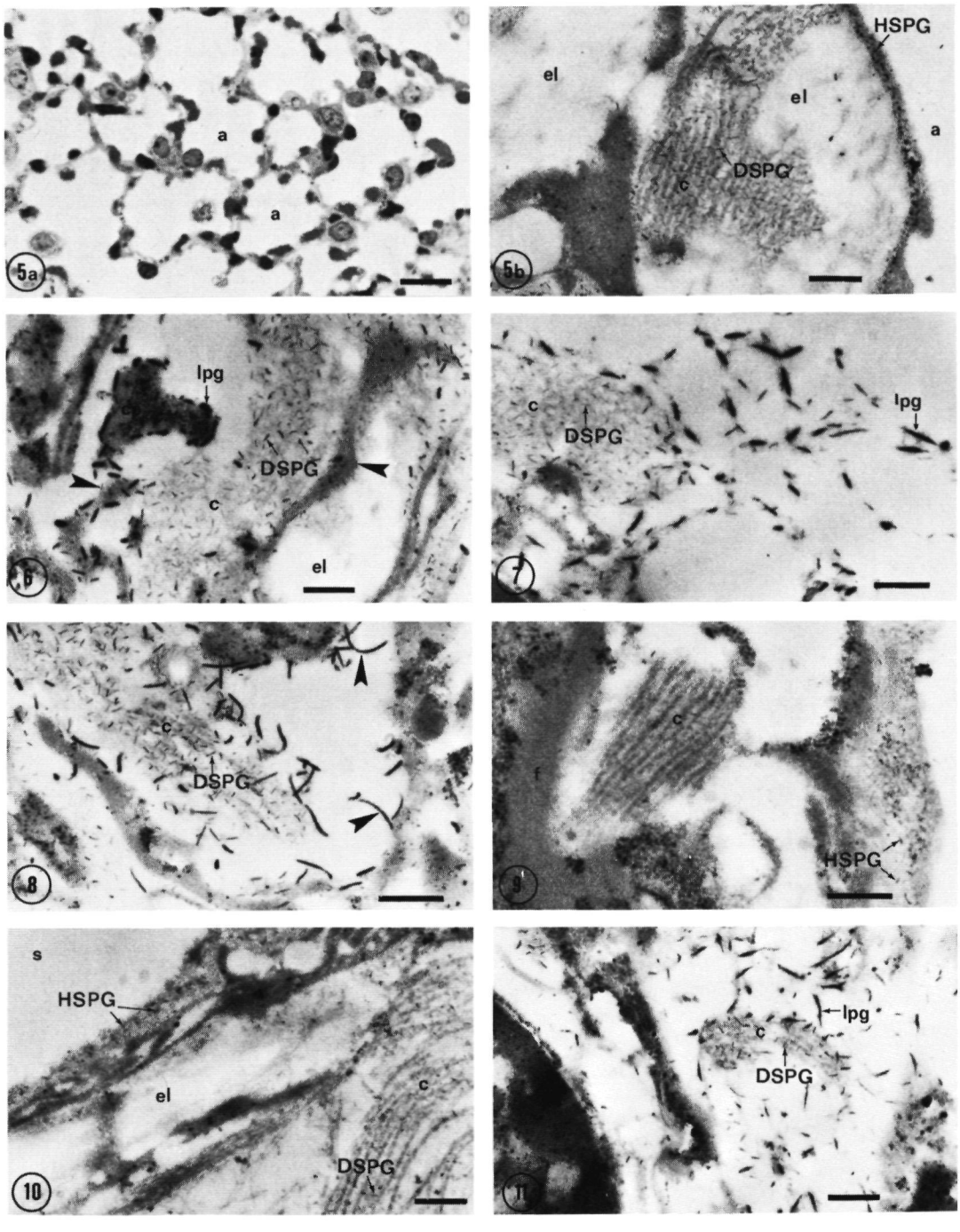
Fig. 7. Mouse lung, day 2.
Large proteoglycans (lpg), associated with non-identified extracellular matrix material, lie in areas which are otherwise devoid of any electron microscopically visible structures.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 8. Mouse lung, day 2.
In the periphery of the developing mouse lung extraordinary large proteoglycans (large arrows) are present. They are located at the boundary between electron microscopically empty areas and extracellular matrix structures or cells.
Poststained with uranyl acetate. Bar: 0.4 µm.

Fig. 9. Mouse lung (day 2) after digestion with chondroitinase ABC (2 U/ml) for 2 h.
Large proteoglycans and collagen-associated dermatan sulfate proteoglycans are no longer visible. Basement membrane-associated heparan sulfate proteoglycans (HSPG) are not affected.
Poststained with uranyl acetate. Bar: 0.3 µm.

Fig. 10. Mouse lung (day 2) after digestion with chondroitinase AC (2/ml) for 2 h.
Large proteoglycans are not detectable; collagen-associated dermatan sulfate proteoglycans (DSPG) are affected and have sometimes disappeared. The basement membrane-associated heparan sulfate proteoglycans (HSPG) are not affected. The epithelial cell layer has disappeared during tissue processing.
Poststained with uranyl acetate. Bar: 0.4 µm.

Fig. 11. Mouse lung (day 2) after treatment with nitrous acid for 22 h.
Large proteoglycans (lpg) and collagen-associated dermatan sulfate proteoglycans (DSPG) are not affected.
Poststained with uranyl acetate. Bar: 0.3 µm.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Characterization of the large PG. The results of the enzyme and nitrous acid treatment are summarized in Table I. The large PG appears to be extremely sensitive to chondroitinase AC and chondroitinase ABC. In all stages of mouse lung development this PG disappears completely when digested with either one of these enzymes. No difference can be observed in its susceptibility to chondroitinase AC or chondroitinase ABC even when used at a concentration of 0,25 U/ml for 1 hr. Overnight treatment with nitrous acid has no influence on the stainability of this large PG.

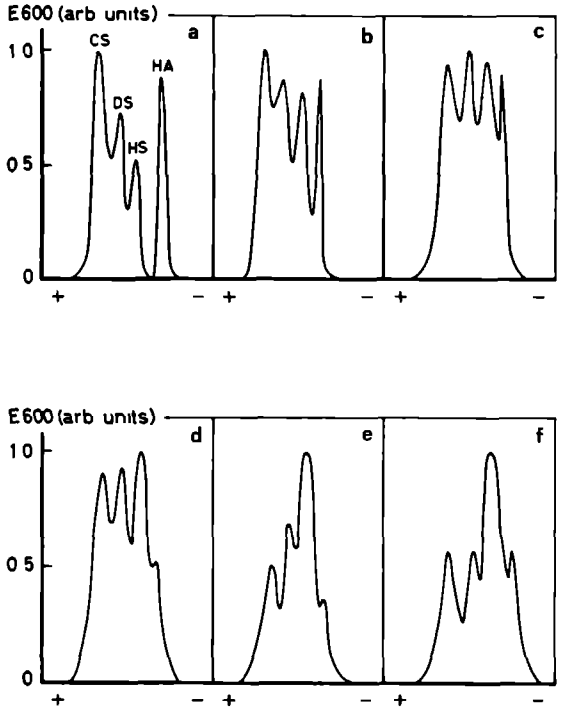

Fig. 12. Cellulose polyacetate electrophoresis of glycosaminoglycans (GAGs) extracted from mouse lungs at various stages of development.
a) standard GAGs. CS: chondroitin sulfate; DS: dermatan sulfate; HS: heparan sulfate; HA: hyaluronic acid. 0.5 µg of each GAG was applied.
b, c, d, e, f: GAG-composition of lungs from day 17 of gestation and day 2, 6, 10 and 27 postnatally, respectively.

Table I. Incubation time and concentrations of the enzyme and nitrous acid treatments, and their effect on the collagen associated, basement membrane associated and large PG from all stages of mouse lung development examined.

			Effect in all stage on:		
Treatment	incubation time	concentration	collagen associated PG	Basement membrane associated PG	Large PG
Chondroitinase ABC	1 h	1/4 U/ml	+	-	+
	1 h	1 U/ml	+	-	+
	2 h	2 U/ml	+	-	+
	22 h	2 U/ml	+	-	+
Chondroitinase AC	1 h	1/4 U/ml	±	-	+
	1 h	1 U/ml	±	-	+
	2 h	2 U/ml	±	-	+
	22 h	2 U/ml	±	-	+
Nitrous acid	22 h	*	-	+	-

The substrates of : chondroitinase ABC are chondroitin sulfate and dermatan sulfate
: chondroitinase AC are chondroitin sulfate, hyaluronic acid and dermatan sulfate where it contains a glucuronic acid residue
: nitrous acid are heparan sulfate and heparin.

-: no effect ±: stainability of PG partially lost +: PG no longer visible

*: nitrous acid was prepared emanating from 5% $NaNO_2$ and 33% HAc (Van Kuppevelt et al., 1984b)

It is widely accepted that the elements of the ECM play an important role in tissue processes such as healing and development (Hay, 1978, 1981; Dolynchuk and Bowness, 1981; Cintron et al., 1978). Although the role of the PGs and GAGs in these processes has achieved much attention, studies on their location and characterization in healing and developing tissues are scarce.

Enzyme digestion studies (Table I) indicate that the large PG observed in developing mouse lung contains CS and/or DS which is rich in glucuronic acid residues. This large CS/DS PG is restricted to the lung connective tissue where it is mainly located at the boundary between ECM structures (e.g. bundles of collagen) and areas which seem electron microscopically empty. Sometimes large PGs are also seen at the surface of cells.

In shape and localization the large CS/DS PG found in developing mouse lung resembles the earlier described large CS/DS PG of human lung (Van Kuppevelt et al., 1985b). However, there are some differences between the locations of both PGs; the large CS/DS PG in developing mouse lung is not or hardly found to be associated with a basement membrane, while the human lung CS/DS PG is seldom or not seen at cell surfaces (Van Kuppevelt et al., 1985b).

We suggest that the large CS/DS PG of developing mouse lung has a large protein core (Van Kuppevelt et al., 1984b), just as that of the adult human lung, and that its rather thick appearance and its heavily staining point to the presence of many GAG chains. It may therefore be able to occupy large hydrodynamic volumes.

The CS/DS PG may have a specific function in developing mouse lung since it

is most abundant in the period between 17 days of gestation and 10 days postnatal with a maximum around 2 days postnatal and since it is not present in the lungs of young adult mice (Van Kuppevelt et al., 1984a,b). Its function may be correlated with processes which are prominent at that time. In developing rat skin, large CS/DS PGs have been detected their concentration decreasing 4-fold within a few days (Habuchi et al., 1986).

The association of the large CS/DS PG with ECM structures indicates its involvement in the proper formation and deposition of the ECM. Especially its association with individual collagen fibrils in the late fetal stage, and its location around bundles of collagen fibrils suggests a commitment in the deposition of these fibrils. This idea is supported by the fact that in rabbit lung the collagen synthesis sharply increases in late fetal life, and although the rate of synthesis starts to fall off before term, it is not until the young adult stage before the collagen synthesis has reached a constant low level (Bradley et al., 1974).

In developing rat lung, fibroblasts show a maximal proliferation at day 4 postnatal and their proliferation rate remains high till 10 days postnatal (Kauffman et al., 1974). At this stage of rat lung development, cell migration must be of considerable importance. Supposing that a similar phenomenon occurs in mouse lung and taken into consideration that the main location of the CS/DS PG is at the boundary of ECM structures and that it occupies a large hydrodynamic volume, the CS/DS PG may mask ECM structures and prevent other structures (e.g. cell processes) from making extensive contact with the ECM. Exogenous CSPG inhibits the fibronectin mediated attachment of fibroblasts to a collagen matrix by binding to collagen or fibronectin (Brennan et al., 1983, Knox and Wells, 1979; Rich et al., 1981). The attachment-inhibiting action of the CS/DS PG may be of importance for the

regulation of cell migration. It has been suggested that this process occurs in a direction of increasing attachment, the velocity of migration falling off as the extent of cell attachment increases (Hay, 1981; Sugimoto and Hagiwara, 1979; Pouysségur and Pastan, 1979). By covering the electron microscopically empty areas the CS/DS PG may constitute a path for cell migration and prevent cells from premature attachment. The cells may ultimately accumulate at places devoid of CS/DS PGs. In the interstitium of developing rat lung the occurence of GAGs in open spaces have also been observed as well as their disappearance when these areas are filled up (Sannes, 1980, 1986). An increase in CS/DS PGs concomitant with cells migration has been noted (Kinsella and Wight, 1986). The occasionally observed cell surface-associated CS/DS PGs may promote the cells' interaction with the ECM, but only when this is not prevented by the CS/DS PGs covering these ECM structures.

Hyaluronic acid (HA), a GAG characterized by a very large hydrodynamic volume (Comper and Laurent, 1978; Gibbs et al., 1968) and often present in large amounts in the early developmental stages of many tissues has also been associated with cell migration phenomena (Toole, 1981, 1982, et al. 1984). Sometimes it is noted that the HA content diminishes as the CS content increases or stays the same in a developing tissue (Toole and Trelstad, 1971; Toole, 1981). Our GAG analyses of the several stages of mouse lung development point in the same direction. We suggest that HA plays a role in the cell migration during the early stages of tissue development, but as development proceeds, and the need for a more refined control of cell migration is required, the 'smaller' CS/DS PG is used which can exert its effect more locally.

Cellular and biochemical mechanisms associated with tissue development are frequently similar to those associated with tissue healing (Brody, 1980; Grant et al., 1983; Thet et al., 1983; Toole, 1981). If a

large CS/DS PG plays a prominent role in a developing tissue, the same large CS/DS PG may be present in a healing tissue. The large CS/DS PG observed in adult human lung and supposed to be involved in healing phenomena (Van Kuppevelt et al., 1985b), and the CS/DS PG found in developing mouse lung might be an example of such a congruence. In this connection it is noteworthy to mention that Hay (1978) described the presence of a large PG in opaque embryonal chicken cornea compared with smaller PG granula in more mature cornea, while Hassell et al. (1983) found an unusually large CS/DS PG in healing rabbit cornea which disappeared when healing was completed.

The large CS/DS PGs we found in developing mouse lung and adult human lung, are probably synthesized by fibroblasts. Large glucuronic acid-rich DSPGs have been detected both in the medium and at the cell surface of embryonal human skin fibroblasts in culture (Cöster et al., 1984). Embryonic human lung fibroblasts in culture synthesize a large CSPG (Vogel and Peterson, 1981).

In conclusion we suggest that the CS in the developing mouse lung manifests itself at least in part in the shape of a large CS/DS PG. This CS/DS PG is restricted to the connective tissue of the lung, where it is located at the boundary of large ECM structures and at the cell surface. From its location and time of presence we deduce that it is associated with processes such as cell migration and cell attachment. The universality of these processes in development and healing suggests that large CS/DS PGs may be characteristic components of healing and developing tissues.

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CHAPTER 9

Proteoglycans, glycosaminoglycans and glycosaminoglycan-degrading enzymes in non-emphysematous and emphysematous human lung parenchyma.

Toin H.M.S.M. VAN KUPPEVELT, HENRIET M.J. JANSSEN, ANDRÉ S.M.J. DOVEREN, JAN-PAUL M.M. BEEKMAN AND CORNELIS D. LAROS

Submitted.

Abstract

Proteoglycans, glycosaminoglycans and glycosaminoglycan-degrading enzymes were studied in specimens of non-emphysematous and emphysematous human lung parenchyma. Glycosaminoglycans constitute about 0.3% of the dry, defatted, peripheral lung tissue. Dermatan sulphate and heparan sulphate are the major glycosaminoglycans, followed by hyaluronic acid; chondroitin sulphate is present only in small amounts. When comparing specimens from non-emphysematous and emphysematous lung parenchyma, no significant differences could be observed in the contents of protein and uronic acid and the glycosaminoglycan composition This was also the case for the activities of a set of exoglycosidases involved in glycosaminoglycan degradation. Ultrastructurally, no differences could be observed in the type and the location of proteoglycans in the alveolar walls of emphysematous and non-emphysematous lungs.

Introduction

Connective tissue plays an important role in the maintenance of the structural integrity of the lung It accounts to a great deal for the mechanical behaviour of the lung during the respiratory cycle. A major non-fibrillar constituent of the extracellular matrix are the PGs* and GAGs. In lung, all types of GAGs have been demonstrated (Motomiya et al., 1982; Horwitz et al., 1976). In lung alveoli, HSPGs have been demonstrated in basement membranes, while DSPGs were found associated with collagen fibrils (Vaccaro & Brody, 1979; 1981; Van Kuppevelt et al., 1985a). Their location and their association with other extracellular matrix components make it likely that PGs (GAGs) play a role in the functioning of the connective tissue skeleton and basement membranes.

*Abbreviations: PG: proteoglycan; GAG: glycosaminoglycan; CS: chondroitin sulphate; DS: dermatan sulphate; HS: heparan sulphate; HA: hyaluronic acid;

There is a general agreement that in emphysema a malfunctioning of the connective tissue skeleton occurs. Although most attention has been focussed on the fibrillar components (viz. elastin and collagen), non-fibrillar components should also be considered. Laros and associates (1972a,b; 1978) stressed the importance of GAGs (PGs) in the pathogenesis of emphysema. A defect in the constitution of GAGs (PGs) could result in a malfunctioning of the extracellular matrix and ultimately lead to emphysematous conditions. Several papers on the involvement of GAGs (PGs) in emphysema have been published (e.g. Karlinsky, 1982; Karlinsky et al., 1983; Lafuma et al., 1985; Radhakrishnamurthy et al., 1985; Konno et al., 1982). From these studies, however, no general pictures emerges on the concernment of PGs (GAGs) in emphysema.

In this study PGs and GAGs were studied, biochemically as well as ultrastructurally, in specimens obtained from non-emphysematous and emphysematous human lung. Furthermore, exoglycosidases, involved in the catabolism of GAGs were investigated.

Materials and methods

Materials

Hyaluronic acid from umbilical cord (grade I), chondroitin-4-sulphate from whale cartilage, dermatan sulphate from porcine skin, heparan sulphate from bovine kidney, bovine serum albumine (fraction V), Streptomyces hyaluronidase, p-nitrophenyl-N-acetyl-ß-D-glucosaminide, 4-methylumbelliferyl-N-acetyl-ß-D-galactosaminide, 4-methylumbelliferyl-ß-D-glucuronide, and 4-methylumbelliferon were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4-methylumbelliferyl-α-L-iduronide and 4-methylumbelliferyl-α-D-glucosaminide were from Behring Diagnostics, (La Jolla, CA, U.S.A.) and p-nitrophenylphosphate and p-nitrophenol were from Merck-Schuchardt (Darmstadt, F.R.G.). Heparitinase (EC 4.2.2.8) from Flavobacterium heparinum was from Seikagaku Kogyo Co., (Tokyo, Japan). Alcian Blue 8GX and Triton X-100 were obtained from Janssen Chimica, (Beerse, Belgium). Cellulose polyacetate electrophoresis was carried out on 25.5 * 14.5 cm cellulose polyacetate strips (Schleicher &

Table I. Preoperative lung function of non-emphysematous and emphysematous patients.

Parameter	Non-emphysematous patients (n=17)	Emphysematous patients (n=17)
Predicted vital capacity (in l) on the basis of height, sex and age	4300 ± 470	3910 ± 205
Actual vital capacity in % of predicted vital capacity	102 ± 12	101 ± 17
Residual volume in % of total lung capacity	31 ± 3	45 ± 4[a]
Forced expiratory volume per sec as % of vital capacity	73 ± 5	44 ± 10[a]
Forced inspiratory volume per sec as % of vital capacity	94 ± 4	87 ± 5
Elastance (in kPa) in relation to functional residual volume	1.32 ± 0.15 (n=3)	0.62 ± 0.16[a] (n=13)

Values are means ± S.D.. For statistic analysis the Student t test was applied:
[a] different from non-emphysematous patients with $P \leq 0.01$

Schull, Dassel, F.R.G.) in a semi-microelectrophoresis chamber obtained from Gelman Instrument Co. (Ann Arbor, MI, U.S.A).

Lung tissue

Lung samples were taken after lobectomy or pneumonectomy for bronchial carcinoma from peripheral, subpleural parts of human lung. The lung tissue was frozen into liquid N_2 and stored at -20°C. Grouping into non-emphysematous and emphysematous persons was done on clinical, radiological, pathophysiological and histological grounds as described by Laros et al., (1972). All persons were men; the average age (± S.D.) of the non-emphysematous group was 62 ± 10, that of the emphysematous group was 64±7. Data on preoperative lung function of this group of patients are given in Table I.

Extraction of glycosaminoglycans

Lung specimens were treated with aceton for 24 h at -20°C, dried above $CaCl_2$ and (after grinding) further defatted in ether/methanol (1:1) for 48 h (4°). Dried samples were suspended in 40 vol. of 0.75 M NaOH

containing 10 mM $NaBH_4$ and allowed to stand for 1 h at 73°C followed by chilling and neutralizing with 10 M HCl. Ice-cold 20% trichloroacetic acid was then added to a final concentration of 5%. After 1 h on ice and centrifugation (15 min, 2000 g), the resulting pellet was washed once with 5% trichloroacetic acid and centrifuged again. The supernatants were pooled and 3 vol. of cold ethanol saturated with sodium acetate was added. After 24 h at 4°C the mixture was centrifuged (15 min, 2000g), the pellet washed once with 80% ethanol (2 h, -20°C) and centrifuged again. The resulting pellet, representing the GAGs, was dried and dissolved in a appropiate volume of deionized water.

Analysis of protein and glycosaminoglycans

Protein was determined by the method of Lowry et al., (1951), using bovine serum albumin as a standard. Uronic acid was assayed according to the method of Blumenkrantz et al., 1973, using D-glucuronic acid as a standard.

For the qualitative analysis of GAGs and the quantitative analysis of DS, the GAGs were electrophoretically separated on cellulose acetate strips using copper acetate as buffer (Stevanovich & Gore, 1967). Strips were stained in a 25 mM NaAc solution containing 50% ethanol, 0.2% Alcian Blue 8GX and 0.05 M $MgCl_2$; destaining was in the same solution without Alcian Blue (Bartold & Page, 1985). Strips were made transparent with paraffin oil and scanned densitometrically at 600 nm using a Zeiss PMQ II spectrophotometer. For the quantification of DS, HS was eliminated prior to electrophoresis, because the HS-band partially overlaps the DS-band. HS was degraded by treatment of the GAG solution for 2 h with HNO_2, made by mixing 5% $NaNO_2$ with 33% HAc (1:1 v/v). DS was quantitated by measuring the surface occupied by the DS-band and comparing it with that of standard DS.

HA and HS were quantified by digestion by specific lyases and determination of the resulting 4,5-unsaturated oligosaccharides by measuring the absorption at 232 nm (Yosizawa et al., 1983).

For HA, 40 µl of a GAG-solution (corresponding to about 10 µg GAG) was mixed with 45 µl 0.1 M NaAc-0.15 M NaCl buffer (pH 5.4) containing 6.5 U Streptomyces hyaluronidase. After incubation for 17 h at 37°C, the reaction was stopped by heating for 2 min. in boiling water. After

chilling, 600 µl of 0 05 M HCl was added and the mixture was centrifuged for 30 min. at 5000 g and 4°C, absorbance was read at 232 nm.

For HS determination, 20 µl of a GAG-solution (corresponding to about 10 µg GAG) was mixed with 30 µl 0.1 M NaAc buffer (pH 7.0), containing 10 mM $Ca(Ac)_2$ and 0.5 U heparitinase. After incubation for 17 h at 43°C, heating in boiling water and chilling, 10 µl containing 20 µg HS was added to obtain precipitation of proteins (Yosizawa et al., 1983). After addition of 600 µl 0.05 M HCl and centrifugation, the absorbance at 232 nm was determined.

The GAG composition in percentages was calculated by the determination of the relative portion of each GAG (i.e. HA, HS and DS) of their sum. CS, which is below 10% was not taken into account.

Exoglycosidases and acid phosphatase

Extraction

To about 0.5 g lung tissue, 10 vol. of 0.05 M citric acid/Na_2HPO_4 buffer (pH 4.2) containing 0.1% Triton X-100 was added Samples were mixed with quartz-sand in a chilled mortar and grinded. After extraction for 50 min, the material was centrifuged for 30 min at 4°C at 23000 g. The supernatant (=extract) was used in the enzyme assays. It was established that 95% or more of the enzymes was extracted using this procedure. Furthermore, the presence of a cocktail of protease inhibitors containing 0.1 M 6-amino-n-caproic acid, 5 mM benzamidine HCl, 1 mM phenylmethylsulphonyl fluoride, 10 mM disodium EDTA, 1 mM iodoacetamide and 10 mM N-ethyl-maleimide was tested. The presence of this cocktail during the extraction procedure and the subsequent enzyme assays did not influence the results. This indicates that no significant degradation of the exoglycosidases or acid phosphatase by proteolytic enzymes occurred.

Enzyme assays

Optimal substrate concentration and pH were used for the assays of exoglycosidases. Incubation conditions for acid phophatase were according to Taga & Van Etten (1982). In all cases product formation

was proportional with the amount of extract

ß-D-N-acetylglucosaminidase (EC 3.2.1.52)

25 µl extract was mixed with 25 µl 0.05 M citric acid/Na_2HPO_4 (pH 4.5) and 50 µl 14 mM p-nitrophenyl-N-acetyl-ß-D-glucosaminide. The final pH was 4.5. After incubation (5 min, 37°C), 0.6 ml 0.2 M glycine/0.2M NaCl (pH 10.7) was added, followed by 2 ml deionized water. The liberated p-nitrophenol was spectrophotometrically measered at 400 nm using a Zeiss PMQ II spectrophotometer.

ß-D-N-acetylgalactosaminidase (EC 3.2.1.52)

25 µl extract was mixed with 25 µl 0.05 M citric acid/Na_2HPO_4 (pH 4.2) and 50 µl 2 mM 4-methylumbelliferyl-N-acetyl-ß-D-galactosaminide. The final pH was 4.3. After incubation (5 min, 37°C), 0.6 ml 0.2 M glycine/0 2 M NaCl (pH 10.7) was added, followed by 2 ml deionized water. The liberated 4-methylumbelliferon was detected fluorimetrically using a excitation wavelength of 363 nm and a emission wavelength of 452 nm. A Zeiss PMQ-3 was used.

ß-D-glucuronidase (EC 3.2.1.31)

25 µl extract was mixed with 25 µl 0.05 M citric acid/Na_2HPO_4 - 0.8 M NaCl (pH 4.3) and 50 µl 2.0 mM 4-methylumbelliferyl-ß-D-glucuronide. The final pH was 4.4. Incubation was for 45 min at 37°C. The liberated 4-methylumbelliferon was measured as described above.

α-L-iduronidase (EC 3.2.1.76)

25 µl extract was mixed with 25 µl 0.05 M citric acid/Na_2HPO_4 - 0.8 M NaCl (pH 3.3) and 50 µl 0.5 mM 4-methylumbelliferyl-α-L-iduronide. The final pH was 4.0. Incubation was for 3 h at 37°C. Measurement of the liberated 4-methylumbelliferon was as described above.

α-D-N-acetylglucosaminidase (EC 3.2.1.50)

25 µl extract was mixed with 25 µl 0.05 M citric acid/Na_2HPO_4 - 0.8 M NaCl (pH 4.0) and 50 µl 0.2 mM 4-methylumbelliferyl-α-D-glucosaminide. The final pH was 4.3. Incubation was for 4 h at 37°C. Measurement of liberated 4-methylumbelliferon was as described above.

Acid phosphatase (EC 3.1.3.2)

25 µl extract was mixed with 25 µl citric acid/Na_2HPO_4 (pH 5.0) and 50 µl 10 mM p-nitrophenylphosphate. The final pH was 5 0. Incubation was for 30 min. at 37°C. The formed p-nitrophenol was determined as described above.

Determination of specific activity

Because of the presence of a considerable and varying amount of blood in the extracts, it was necessary to make a correction for the protein derived from blood. For this purpose, the hemoglobin content in the extract was determined according to a slightly modified method of Kampen & Zijlstra, 1961. Briefly, 100 µl extract was mixed with 900 ul 0.2 M phosphate buffer (pH 7.2), containing 1.0 mM KCN, 0.6 mM $K_3Fe(CN)_6$, 1.5 mM NaCl and 0.05% Triton X-100. After 3 min, the absorbance at 546 nm was read. When the amount of hemoglobin is known, the total amount of blood proteins can be calculated by multiplication with a factor of 1.25. The amount of protein derived from the lung tissue itself was calculated by subtracting the amount of blood protein from the total amount of protein.

It was established for ß-D-N-acetyl-glucosaminidase, ß-D-N-acetyl-galactosaminidase, α-L-iduronidase and ß-D-glucuronidase that the activity in blood was negligible compared with the enzyme activity from the lung tissue itself. The α-D-N-acetylglucosaminidase and the acid phosphatase activity in blood, however, could not be neglected and were subtracted from the total activities.

Electron microscopy

To investigate proteoglycans at the ultrastructural level, the Cuprolinic Blue staining procedure was used as described previously (Van Kuppevelt et al., 1985a). Sampling and the use of chondroitinase ABC, chondroitinase AC and nitrous acid were also as described.

Results

In non-emphysematous human lung parenchyma, the amount of uronic acid is 1.26 µg/mg dry defatted tissue (Table II). This corresponds to about 3 µg GAG/mg dry defatted tissue. The values were not corrected for blood components. Using cellulose acetate electrophoresis and/or enzyme digestions, it was found that DS and CS are the major GAGs in the peripheral lung tissue, followed by HA. CS is present only in small amounts (less than 10%). Heparin could not be detected. When comparing non-emphysematous with emphysematous specimens, no

Table II. Protein and uronic acid content and glycosaminoglycan composition of non-emphysematous and emphysematous lung tissue.

Constituent	Non-emphysematous tissue	Emphysematous tissue
	(n=11)	(n=11)
Protein	733 ± 43	758 ± 36
Uronic acid	1.26 ± 0.27	1.28 ± 0.47
Ratio uronic acid/protein	1.72 ± 0.37	1.57 ± 0.51
	(n=10)	(n=9)
Hyaluronic acid	24 ± 11	27 ± 10
Heparan sulphate	38 ± 7	35 ± 7
Dermatan sulphate	38 ± 14	38 ± 9

Protein and uronic acid content are expressed as mg/g delipidated, dry lung tissue. Glycosaminoglycans are given in % of total content; chondroitin sulphate, which is below 10% was not taken into account.
For statistical analysis the two-sided Wilcoxon's rank sum test was used with a confidence level of α=5%. Values are means ± S.D. and are not significantly different for both groups.

Table III. Exoglycosidase and acid phosphatase activities in non-emphysematous and emphysematous lung tissue.

Enzyme	Non-emphysematous tissue	Emphysematous tissue
	(n=31)	(n=19)
β-D-N-acetylglucosaminidase	142 ± 77	183 ± 89
β-D-N-acetylgalactosaminidase	17 ± 14	18 ± 10
Acid phosphatase	84 ± 46	83 ± 46
	(n=15)	(n=14)
β-D-glucuronidase	0.112 ± 0.064	0.124 ± 0.056
α-L-iduronidase	0.035 ± 0.017	0.038 ± 0.015
α-D-N-acetylglucosaminidase	0.018 ± 0.009	0.021 ± 0.010

Enzyme activities are expressed as nmoles of substrate hydrolyzed/min per mg protein. For statistical analysis the two-sided Wilcoxon's rank sum test was used with a confidence level of α=5%. Values are means ± S.D. and are not significantly different for both groups.

significant differences could be detected in the content of protein and uronic acid and the GAG composition (Table II).

In alveoli of non-emphysematous lung, PGs are periodically associated with collagen fibrils (Fig. 1a) These PGs were chondroitinase AC- and nitrous acid-resistant, but could be degraded with chondroitinase ABC; they therefore represent DSPGs, as described earlier (Van Kuppevelt et al., 1985a). The PGs present in the alveolar and capillary basement membrane (Fig. 1b) were sensitive towards nitrous acid, but not towards chondroitinase ABC and chondroitinase AC, indicating their HSPG-nature. Four emphysematous lungs were studied ultrastructurally for the presence of PGs in the alveoli (Fig 1c). When compared with normal alveoli, no differences could be detected in the location of the PGs and their sensitivity towards GAG-degrading procedures. However, no morphometric studies were performed.

Finally, the specific activity of a set of exoglycosidases was studied (Table III) No significant differences between specimens from non-emphysematous and emphysematous lungs could be observed. Individual variation, however, is considerable. In an attempt to reduce this variation, enzyme activities were related to the activity of the universal lysosomal enzyme acid phosphatase. It was reasoned that a variation in enzyme activity due to local processes (e.g. inflammatory cells) could be met in this way. The specific activity of acid phosphatase did not differ significantly between specimens from non-emphysematous and emphysematous lungs (Table III). Although in some cases the coefficient of variation was substantially reduced by relating exoglycosidases to acid phosphatase, this was not a general pattern. When specimens of non-emphysematous and emphysematous lungs were compared, no significant difference could be detected in enzyme activities related to acid phosphatase (data not shown).

Discussion

The GAG content found in non-emphysematous human lungs is about 0.3% of the defatted dry weight, a value corresponding to that found by others (Konno et al., 1982; Schmid et al., 1982). The amount of GAG derived from blood accounts for only about 0.2% of the lung GAGs

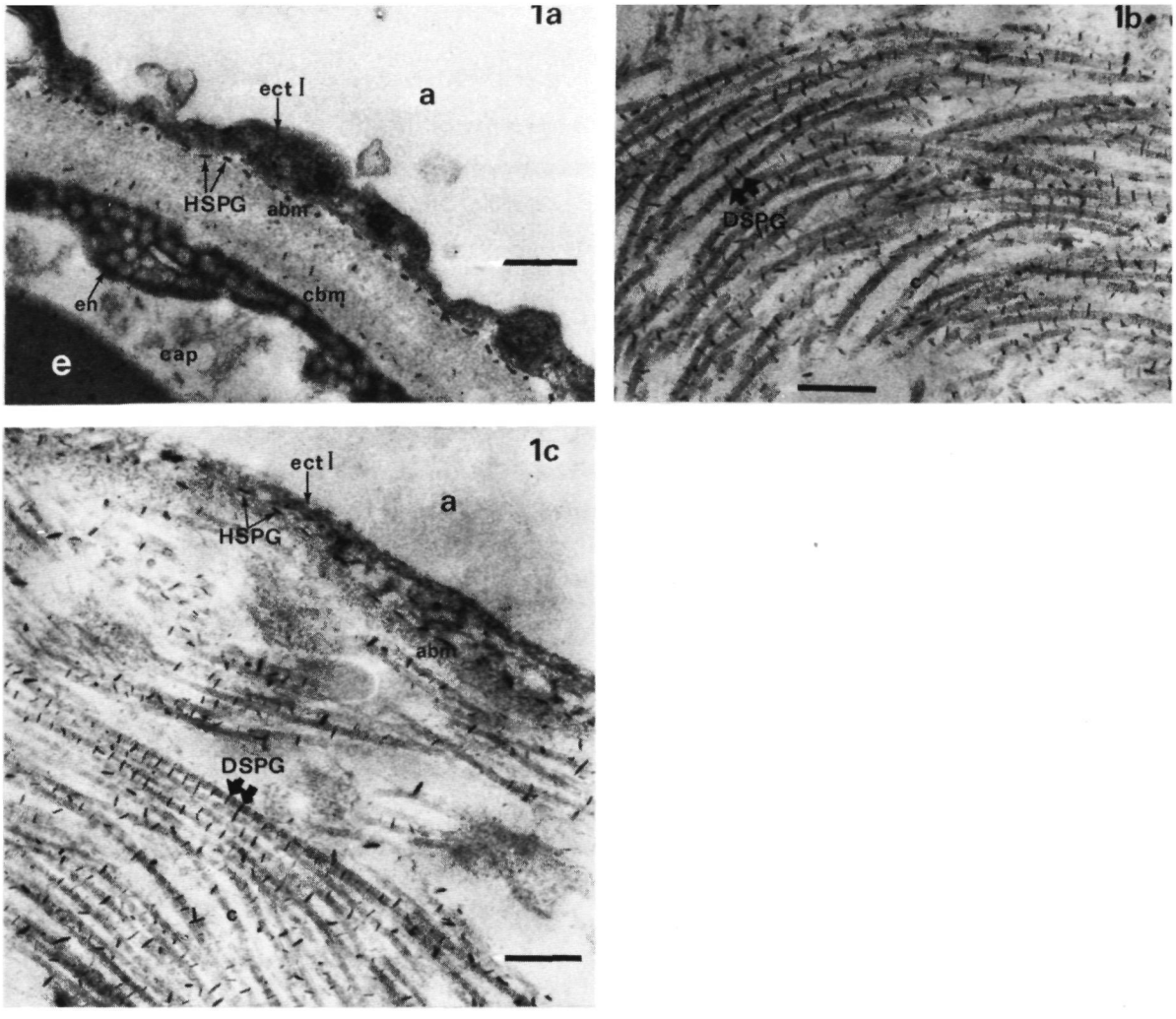

Fig. 1. Part of an alveolar wall after the Cuprolinic Blue staining procedure, used to detect proteoglycans. Bar: 300 nm. Poststained with uranyl acetate and lead citrate.
a,b: non-emphysematous lung.
a. Heparan sulphate proteoglycans (HSPG) are located in the alveolar basement membrane (abm) as well as in the capillary basement membrane (cbm).
b. Dermatan sulphate proteoglycans (DSPG) are regularly associated with collagen fibrils (c) and are separated from each other according to the main banding period of the collagen fibrils (about 60 nm).
c: emphysematous lung. The form and location of the HSPGs and the DSPGs are similar when compared to the non-emphysematous lung.
a: alveolar airspace; cap.: capillary; ect I: type I epithelial cell; en: endothelium.

(Schmid et al., 1982). Of the 5 types of GAGs, HA, CS, DS, and HS could be detected in peripheral lung tissue. The two main GAGs, DS and HS, are present in the alveoli as PGs and are associated with collagen fibrils and basement membranes, respectively (Van Kuppevelt et al., 1985a; Fig. 1). In an elastase-induced emphysema model, alveolar edema coincides with a loss of HSPGs and edema subsides when regeneration occurs (Vaccaro et al., 1985). This points to a filter function for the HSPGs as suggested (Van Kuppevelt et al., 1984). CS, a minor GAG in our preparations, may be present (at least partially) as the large CS/DSPG, which are irregularly present in human lung (Van Kuppevelt et al., 1985b). Heparin could not be detected in human lung parenchyma, in accordance with the results of Schmid and coworkers (1982). However, since mast cells are present, small amounts of this GAG are likely present. Keratan sulphate is not present in peripheral lung tissue: it is restricted to the tracheobronchial cartilage (Mason & Wusteman, 1970).

Several studies have been published dealing with the possible involvement of PGs (GAGs) in emphysema. The outcome of these studies is often contradictory. For human emphysematous lung a decrease and an increase in the percentage HA have been noted (Konno et al., 1982; Pecora et al., 1966). An increase in the glucosamine/galactosamine ratio was detected for human emphysematous lung by Laros et al., (1972), but Saltzman and coworkers (1961) did not demonstrate such an increase. In pronase-induced emphysema, an increase in HA and DS was noted (Radhakrishnamurthy et al., 1985), while elastase-induced emphysema was characterized by an elevated incorporation of [^{14}C]glucosamine into HS and HA (Lafuma et al., 1985). Karlinsky (1982), also working with elastase-induced emphysema demonstrated an increase in DS, but could not corroborate this result later (Karlinsky et al., 1983). In starvation-induced emphysema, no change in the amount of GAGs could be detected (Karlinsky et al., 1986). Studies are difficult to compare due to different methodologies. Furthermore, the type of emphysema seems to be of importance. In rabbits, $CdCl_2$-induced centrilobular emphysema was characterized by a decrease in uronic acid, while an increase was found in pronase-induced panlobular emphysema (Radhakrishnamurthy et al., 1985).

In this study no significant differences could be observed in the

content of protein and uronic acid in specimens from non-emphysematous and emphysematous lungs, in accordance with the results of Laros et al., (1972). Their suggestion of an internal shift in the composition of the GAGs, however, could not be confirmed. Furthermore, the activities of exoglycosidases involved in the catabolism of GAGs were similar for the emphysematous and the non-emphysematous group. It should be noted that there is a large individual variation. A considerably larger number of lung specimens should be investigated in order to make a more definite statement. In all 4 emphysematous lungs examined ultrastructurally, no differences could be detected in the location of the PGs nor in their sensitivity towards GAG-degrading procedures.

Another point should be considered here. From studies on elastase-induced emphysema, it has become clear that the time of tissue sampling is of importance Incorporation of [^{14}C]glucosamine into GAGs was initially lower after elastase administration, but then increased (together with the total GAG level); at day 21, the situation returned to normal and stayed that way over the period measured (1 year), although progression of airspace enlargement and distortion still continued (Karlinsky et al., 1983). In explant cultures of hamster lungs, incorporation of [^{14}C]glucosamine into HS and HA was less at day 24 after elastase administration than at day 1 (Lafuma et al., 1985) In a ultrastructural study, it was noted that HSPGs in the alveolar basement membrane were degraded 2 h after elastase treatment, but that regeneration was well underway at day 4 and was completed at day 10 (Vaccaro et al., 1985). The severity of the emphysema, however, is known to progress for many months after induction (Snider et al , 1986). In men, where emphysema is more or less an end-stage phenomenon, a similar situation may occur. GAGs may be initially affected, but (apparently?) restored at a later time.

An approach in the study on the possible involvememt of PGs (GAGs) in emphysematogenesis, could be trying to provoke emphysema by broncho-alveolar lavage with specific GAG-degrading enzyme.

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CHAPTER 10

Survey and summary

Survey and summary

A main goal of this investigation on proteoglycans and glycosaminoglycans was to get a clue as to which role these components play in lung parenchyma. Since a combination of diciplines may be complementary, both histochemical and biochemical techniques were applied

Cuprolinic staining procedure

In Chapter 2, a staining procedure is described using the cationic dye Cuprolinic Blue in a critical electrolyte concentration method. This procedure detects proteoglycans on basis of their sulphate groups. The specificity of this method was demonstrated by use of enzymes and chemical treatments (Chapter 3). Proteoglycans appear as electron-dense filaments, probably caused by the collapse of the glycosaminoglycan side-chain(s) onto the protein core. Comparison with other studies, makes it acceptable that each filament represents a monomer (i.e. one molecule). X-ray diffraction studies have indicated that no dislocation of proteoglycans occurs during dehydration and embedding (Meek et al., 1985 and 1986). In Chapter 4, an attempt is made to correlate biochemical data from literature with the ultrastructural appearance of proteoglycans. Although one has to be cautious, the appearance may be indicative for the glycosaminoglycan/protein ratio and for the molecular weight of the part of the protein core to which glycosaminoglycans are attached.

In conclusion, the Cuprolinic staining procedure is a suitable method for the ultrastructural localization and characterization of proteoglycans in tissue.

Proteoglycans in lung parenchyma

From electron microscopical investigations of alveoli of mouse (Chapter 3), man (Chapter 5) and cattle (Chapter 6), the picture has emerged that two types of proteoglycans are permanently present: dermatan sulphate proteoglycans, which are associated with collagen fibrils and heparan sulphate proteoglycans, which are confined to basement membranes. Biochemical analysis of the parenchyma (including small bloodvessel and bronchioli) showed that dermatan sulphate and heparan sulphate are the predominant glycosaminoglycans and that

hyaluronic acid is also present in considerable amounts (Chapter 9). Since the latter lacks sulphate groups, its presence c.q. location in alveoli can not be demonstrated by the Cuprolinic Blue staining procedure.

A large, irregularly present, chondroitin sulphate/dermatan sulphate proteoglycan was detected in human alveoli (Chapter 7). A similar proteoglycan was found during a distinct phase of mouse lung development (Chapter 8). It was suggested that this proteoglycan plays a role in tissue repair and development; more evidence, however, is needed to make a more definite statement of its function.

Dermatan sulphate proteoglycan

It was demonstrated that an iduronic acid-rich dermatan sulphate proteoglycan is regularly associated with collagen fibrils in human alveoli, bovine alveoli, small bloodvessels and bronchioli (Chapter 5 and 6). The isolation of this proteoglycan and its biochemical characterization is described in Chapter 6. It is a small proteoglycan with 1-3 dermatan sulphate side-chain(s) and a high content (91%) of iduronic acid residues. On base of morphological and biochemical data, the following model has been proposed for the interaction of the dermatan sulphate proteoglycan with the collagen fibrils: the anionic protein core is specifically attached to the fibrils, while proteoglycans from neighbouring collagen fibrils are attached to each other by the dermatan sulphate side-chains. In this way the fibrils adhere to each other and provide structural coherence. This may cause the collagen fibrils to act together as fibres instead of separate fibrils. For a dynamic organ as the lung, this may be of importance. Recent information indicates that the protein core is necessary for binding of dermatan sulphate proteoglycans to collagen fibrils in vitro (Scott et al., 1986).

The obtained ultrastructural and biochemical data give us a clue to the functions of proteoglycans in lung parenchym. More experimental evidence may be obtained by specific enzymatic digestions of proteoglycans or glycosaminoglycans in vivo and examination of the effect of these treatments on lung mechanics. Elastase has been shown to reach the alveolar interstitium after intratracheal instillation. The transport to the extracellular matrix was predominantly by endo-

and exocytosis (Snider et al., 1986). Collagen fibrils are affected after endotracheal instillation of collagenase (Karlinsky et al., 1976). Using specific glycosidases in perfusion studies, direct evidence was obtained for the role of heparan sulphate proteoglycans in establishing the restrictive permeability of the glomerular basement membrane (Kanwar et al., 1980; Rosenzweig and Kanwar, 1982). In lung, experiments in vivo applying chondroitinase ABC or chondroitinase B, which specifically degrades dermatan sulphate, should provide more conclusive evidence about the function of the dermatan sulphate proteoglycan.

It has been suggested that lysyl oxidase is a proteoglycan conjugate since after chondroitinase ABC treatment 75% of the enzyme activity was destroyed. (Tanzer and Housley, 1981) Lysyl oxidase is extracellularly involved in the cross-linking of collagen and appears to work preferentially on collagen molecules after they have aggregated into fibrillar forms. The number of ruthenium red-positive, collagen fibril-associated granules (dermatan sulphate proteoglycans ?) was reduced in cornea by treatment with beta-aminopropionitrile, an inhibitor of lysyl oxidase (Moorhead, 1981). Whether dermatan sulphate proteoglycans are involved in the cross-linking process by association with lysyl oxidase is intriguing, but needs further clarification.

Heparan sulphate proteoglycans.

Heparan sulphate proteoglycans of human and mouse alveoli are present in alveolar and capillary basement membranes (Chapter 2, 3 and 5) In the alveolar basement membrane they are lying in one plane, thus providing the alveolus with an envelope of negatively charged molecules. A filter function seems likely for these proteoglycans. After intratracheal instillation of elastase in hamsters, a loss of heparan sulphate proteoglycans was noticed, coinciding with alveolar edema (Vaccaro et al., 1985a). As the proteoglycans were regenerating, edema subsided. They therefore may act as a charge barrier and prevent leak of proteins from capillaries into the alveolar airspace. One should, however, be careful since elastase degrades also other molecules such as elastin, collagen and fibronectin. A similar study with heparitinase, which digests specifically heparan sulphate, might be more conclusive. It has been suggested that basement membrane

elasticity contributes to the lung mechanical properties (Vaccaro et al., 1985b). By mutual repulsion of the negative charges, the proteoglycan envelope may be of importance in this respect.

There exists a heterogeneity in the appearance of heparan sulphate proteoglycans in the basement membranes of lung alveoli. In the capillary basement membrane, the proteoglycans are more scattered and stain less intense (i.e. contain less sulphate groups) when compared to the alveolar basement membrane. This may relate to a greater permeability of the capillary basememt membrane, which may be necessary in view of the transendothelial transport. In mice, the alveolar basement membrane underlying type II epithelial cells, contains smaller proteoglycan-filaments than the basement membrane underlying type I epithelial cell.

Proteoglycan-filaments in the alveolar basement membrane of mice are larger compared to those in the human alveolar basement membrane (Chapter 2 and 5). The latter sometimes contain a second layer of proteoglycans, not observed in alveolar basement membranes of adult mouse. The possible fuctional consequences of these differences are not clear.

Emphysema

When comparing specimens from non-emphysematous and emphysematous lungs, no differences could be determined in the amount of uronic acid, the glycosaminoglycan composition and the activity of glycosaminoglycan-degrading enzymes (Chapter 9). Ultrastructurally, the location of proteoglycans and their sensitivity toward degrading procedures is similar in both groups. These results do not exclude the involvement of proteoglycans in the pathogenesis of emphysema, since only advanced stages were examined. An experiment to test this involvement, is to try to provoke emphysema by intratracheal instillation of glycosaminoglycan-degrading enzymes.

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Vaccaro, C.A., Brody, J.S., Snider, G.L. (1985b). Alveolar wall basement membranes in bleomycin-induced pulmonary fibrosis. Am. Rev. Respir. Dis. 132, 905-912.

In dit proefschrift wordt een electronenmicroscopisch en biochemisch onderzoek naar proteoglycanen en glycosaminoglycanen in longparenchym beschreven. Proteoglycanen zijn macromoleculen bestaande uit een eiwitketen waaraan covalent glycosaminoglycanen en oligosacchariden zijn gebonden. De proteoglycanen zijn als gevolg van de talrijke carboxyl- en sulfaat groepen sterk negatief geladen.

In hoofstuk 1 wordt de structuur van de long beschreven. Tevens komen drie belangrijke componenten van de extracellulaire matrix aan de orde: collageen, elastine en proteoglycanen. De relatie van proteoglycanen met emfyseem wordt kort besproken.

In hoofstuk 2 wordt een kleuringsmethode besproken voor het zichtbaar maken van proteoglycanen op ultrastructureel niveau. Hiertoe is de cationische kleurstof Cuprolinic Blue gebruikt volgens de "critische electrolyt concentratie" methode.

De specificiteit van de kleuringsmethode is onderzocht in hoofdstuk 3. Door middel van enzymatische en chemische afbraakprocedures kon aangetoond worden dat de na de kleuring zichtbare electronen-dichte filamenten proteoglycanen vertegenwoordigen; elk filament is waarschijnlijk een molecuul. Met behulp van genoemde procedures kan tevens het type proteoglycaan vastgesteld worden.

In hoofstuk 4 is voor een aantal bindweefsels gepoogd een correlatie te leggen tussen het ultrastructurele voorkomen van proteoglycanen en hun biochemische karakteristieken. Alhoewel grote voorzichtigheid geboden is, kan gesteld worden dat uit morfologische karakteristieken aanwijzingen verkregen kunnen worden omtrent de glycosaminoglycaan/eiwit ratio en het molecuul gewicht van dat deel van het eiwit waaraan glycosaminoglycanen gebonden zijn.

Alveoli van de muis, de mens en het rund zijn ultrastructureel onderzocht op hun proteoglycaan samenstelling (hoofdstuk 3, 5 en 6). Heparaan sulfaat proteoglycanen zijn aanwezig in de basaal membranen, terwijl dermataan sulfaat proteoglycanen geassocieerd zijn met collageen fibrillen. Voor humane en runder alveoli en voor kleine bloedvaatjes en bronchioli van het rund kon aangetoond worden dat het dermataan sulfaat proteoglycaan rijk is aan iduronzuur. De isolatie van dit proteoglycaan uit runder longparenchym wordt beschreven in

hoofdstuk 6. Het is een klein proteoglycaan met 1-3 dermataan sulfaat zijketens met een hoog iduronzuur gehalte (91% van het totale uronzuur). Op grond van ultrastructurele en biochemische gegevens is een model opgesteld voor de interactie tussen dermataan sulfaat proteoglycaan en collageen fibril (hoofdstuk 6). In dit model bindt de negatief geladen eiwitketen specifiek aan de collageen fibril, terwijl proteoglycanen gebonden aan verschillende fibrillen met elkaar verbonden zijn door associatie van dermataan sulfaat ketens. Op deze manier worden collageen fibrillen met elkaar verbonden en kunnen ze gezamenlijk functioneren als vezels
Er bestaat een heterogeniteit in de heparaan sulfaat proteoglycanen in de basaal membranen (hoofdstuk 3 en 5). De proteoglycanen in de capillaire basaal membraan liggen meer verspreid en kleuren minder intens (i.e. bevatten minder sulfaat groepen) dan die in de alveolaire basale membraan. Verder zijn er verschillen aanwezig tussen heparaan sulfaat proteoglycanen in alveoli van mens en muis. De aaneengesloten laag van negatief geladen heparaan sulfaat moleculen in de alveolaire basaal membraan duidt op een filterfunctie. Mogelijkerwijs speelt deze laag ook een rol in de longmechanica.

In humane alveoli werd een groot, onregelmatig aanwezig, proteoglycaan aangetoond. Het handelt zich hier om een chondroitine sulfaat en/of glucuronzuur-rijk dermataan sulfaat proteoglycaan (hoofdstuk 7). Een soortgelijk proteoglycaan is aanwezig tijdens een bepaalde fase van de longontwikkeling in de muis (hoofdstuk 8).

In hoofdstuk 9 is het onderzoek beschreven naar een mogelijke betrokkenheid van proteoglycanen in de longziekte emfyseem. Er kon geen verschil aangetoond worden tussen monsters van emfysemateuze en niet-emfysemateuze humane long wat betreft het uronzuur gehalte en de glycosaminoglycaan samenstelling. De activiteit van een vijftal glycosaminoglycaan-afbrekende enzymen en de ultrastructurele karakteristieken van de proteoglycanen in alveoli vertoonden ook geen duidelijke verschillen.

Toin van Kuppevelt werd op 8 november 1955 geboren te Grave.
In 1974 behaalde hij het Gymnasium β diploma aan het St. Dominicus College te Nijmegen.
Hij studeerde biologie aan de Katholieke Universiteit te Nijmegen, waar hij in 1981 het doctoraal examen deed met als hoofdvak submicroscopische morfologie (o.l.v. Prof. Dr. M.M.A. Sassen) en als bijvakken chemische cytologie (o.l.v. Prof. Dr. Ch.M.A. Kuyper) en cytologie-histologie (o.l.v. Dr. W. Eling en Prof. Dr. C. Jerusalem).
Van 1981 tot 1986 was hij als wetenschappelijk medewerker werkzaam op de afdeling Chemische Cytologie van de Faculteit der Wiskunde en Natuurwetenschappen van de Universiteit Nijmegen. Gedurende deze tijd leverde hij een bijdrage aan het onderwijs van biologie studenten.

STELLINGEN

I
De ultrastructurele locatie van proteoglycanen in situ kan belangrijke aanwijzingen geven omtrent de functie van deze macromoleculen.
Dit proefschrift

II
Het fotografisch bewijsmateriaal dat Vaccaro en Brody overleggen om aan te tonen dat de basaal-membraan van capillaire endotheelcellen in alveoli van de long van de rat niet alleen heparaan sulfaat proteoglycanen bevat, maar ook chondroitine sulfaat- en/of dermataan sulfaat proteoglycanen is niet overtuigend.
Vaccaro, C.A. en Brody, J.S., 1981. J. Cell Biol. 91, 427-437.

III
De controversiele resultaten van Karlinsky en medewerkers omtrent de betrokkenheid van glycosaminoglycanen bij de pathogenese van emfyseem zijn tekenend voor de onduidelijkheid op dit gebied.
Karlinsky, J.B., 1982. Am. Rev. Respir. Dis. 125, 85-88. Karlinsky, J.B. et al., 1983. J. Lab. Clin. Med. 102, 151-162. Karlinsky, J.B. et al., 1986. Am. J. Physiol. 251, R282-R288.

IV
De aanwijzingen die hebben geleid tot de gepostuleerde partiele transformatie van interne matrix fibrillen tot chromosoomscaffolds tijdens de transitie in een eukaryotische celcyclus van interfase naar mitose, zijn overtuigend.
Bekers, A.G.M. et al., 1986. J. Cell Sci. 86, 155-171.

V
Bij de isolatie van lymfocyten uit bloed door middel van centrifugatie over "lymphocyte separation medium", dient men zich ervan bewust te zijn dat dit niet het enige celtype is dat zich na centrifugatie in de interfase bevindt.
Nooij, F.J.M. et al., 1986. Eur. J. Immunol. 16, 981-984.

VI
Het verdient aanbeveling de diffusiecoëfficiënten van substraten in poreuze gelen te relateren aan de concentratie van het dragermateriaal in de deeltjes in plaats van aan de concentratie van het materiaal in de oplossing voor gelbereiding.
Nguyen, A-L. en Luong, J.H.T., 1986. Biotechnol. Bioeng. 28, 1261-1267.

VII
Bij een onderzoek naar een ziekte waarbij patientenmateriaal gebruikt wordt, verdient het de voorkeur de onderzoeker te stationeren in het ziekenhuis waarvan het materiaal betrokken wordt.

VIII
In het openbaar rokende anti-kernenergie demonstranten zijn en blijven -ook na het kernreactorongeval in Tsjernobyl- hypocriet.

IX

Bij sommige bioscoopfilms zou men zich gewenst hebben dat de tekst "In verband met de lengte van het programma valt de pauze tijdens de hoofdfilm" was vervangen door de tekst "In verband met zijn kwaliteit valt de hoofdfilm tijdens de pauze".

Nijmegen, 9 april 1987

Toin van Kuppevelt